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JOURNAL OF THE NATIONAL CANCER INSTITUTE

VOLUME 9 DECEMBER 1948 NUMBER 3

MUCOLYTIC ENZYME SYSTEMS. III. INHIBITION OF HYALURONIDASE BY SERUM IN HUMAN CANCER[1,2,3]

By ERICK Y. HAKANSON, M. D., *junior research fellow,*[4] *National Institutes of Health, Public Health Service,* and DAVID GLICK, Ph. D., *associate professor of physiological chemistry, Medical School, University of Minnesota*

INTRODUCTION

A conceivable factor in the invasiveness of malignant tumors might be the elaboration of a mucolytic principle able to break down the normal connective tissue barriers and thereby help the spread and implantation of the tumor cells. Since hyaluronidase is a potent "spreading factor," and its substrate hyaluronic acid has wide distribution in the animal body, the possible role of this enzyme in malignancy was considered worthy of investigation. Duran-Reynals (*1*) reported that extracts from human epithelial tumors enhanced, had no effect, or inhibited the spread of vaccinia virus and india ink in the rabbit skin, while sarcomas either inhibited or had no effect. It was also reported that certain malignant mouse tumors contained appreciable amounts of a factor that increased the spread of staphylococci infections in rabbit skin (*2*) and caused the liberation of reducing substances from hyaluronic acid (*3*). Boyland and McClean (*4*) attempted to relate the rate of growth of various malignant animal tumors to their concentration of spreading factor and found that there was a rough parallelism for mammalian tumors. However, the activities of tumor tissue extracts were much less than those of testicular extracts. Placenta and embryo contained moderate but variable activities while other normal tissues showed little. Recently McCutcheon and Coman (*5*), using both the spreading in rabbit skin and the viscosimetric assay methods for hyaluronidase activity, were able to demonstrate greater activity in some cases in human carcinomas than in normal control tissues by both methods. The results of our investigations on the hyaluronidase content of human tumors will be reported later.

This problem was also approached experimentally by observing the effect of injected hyaluronidase on the growth or spread of tumors in animals. Unfortunately, the early observations on this aspect of the problem yielded results which were inconclusive and inconsistent (*6–11*). In an abstract of a recent paper by Simpson and Ayengar (*12*), it was reported that hyaluronidase markedly increased the invasiveness of transplantable carcinomas in mice. Coman and co-workers (*13*) were unable to prove that injected hyaluronidase increased the invasiveness of the animal tumors studied.

Another aspect of the possible role of hyaluronidase in malignancy is the effect of naturally occurring inhibitors of the enzyme. Duran-Reynals (*14*) originally reported that the infection enhancing power of testicle extract was neutralized in vivo by an antiserum against homologous testicle extract and that it was species specific. The specific nature of the inhibitor was confirmed when hyaluronidase was measured by the rate of depolymerization (*15*) or hydrolysis (*16*) of hyaluronic acid or by its ability to prevent the dispersion of the follicle cells surrounding rat ova (*17*). Consequently, it was concluded that

[1] Received for publication June 2, 1948.

[2] Aided by a grant from the National Institutes of Health, Public Health Service.

[3] The authors wish to express their appreciation to Page Edmondson, Mrs. Signe Olson, and Mrs. Doris Groten for technical assistance in carrying out this investigation.

[4] Data in this paper were presented in a thesis submitted in partial fulfillment of the requirements of the Graduate School of the University of Minnesota for the degree of Master of Science in Physiological Chemistry.

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a hyaluronidase inhibitor, specific for the species used to supply antigen, exists in blood sera and that normal serum exerts relatively little inhibition. However, McClean (*18*), by use of decapsulation of virulent bacteria as a test for hyaluronidase, reported inhibition by both homologous and heterologous sera. Hobby et al. (*16*) found that rat and rabbit serum inhibited hyaluronidases from different species of bacteria. Haas (*19*) studied the inhibitory effect of normal sera of a variety of birds, fish, and mammals against hyaluronidases from various sources and found that the inhibitory effect was of general occurrence and nonspecific. A number of recent investigations (*20–22*) confirmed the presence of a nonspecific hyaluronidase inhibitor in normal human serum. Thus it appears that in addition to antisera specific for the source of the enzyme, which is used as the antigen, there also exists a nonspecific hyaluronidase inhibitor in blood serum which can effectively inhibit the enzyme from a wide variety of sources. It is conceivable that the latter ever-present blood-serum inhibitor plays a role in the defense of the animal body against the invasive characteristics of the malignant cell. In order to investigate this possibility, the first study undertaken was to determine what variations occurred in the level of this inhibitor in persons afflicted with malignant disease. The present report is concerned with the results of this study.

MATERIALS AND METHODS

The details of the preparation of the materials and the viscosimetric assay method used were given in a previous paper (*25*). The hyaluronidase was prepared from bovine testicle and the hyaluronic acid from human umbilical cord. The unit of hyaluronidase inhibition, A, is defined as:

$$A=\frac{R-R_o}{R_o}$$

where (R_o) is the time in seconds required to reduce the viscosity of the reaction mixture to half its original value, and R is the time in seconds required to reduce initial viscosity to half after incubation of the 0.5 cc. of enzyme solution with 0.02 cc. serum for 10 minutes at 37.5° C. The data were recorded as A/cubic centimeter of the blood serum,

$$\text{i. e. } \frac{R-R_o}{R_o}\times 50.$$

In order to eliminate as many unknown factors as possible, blood was drawn only from those patients who satisfied the following conditions: no previous treatment; no known concurrent disease other than the cancer; no blood transfusion for at least 3 months previously; no temperature above 99.6° F. as the daily maximum at any time of their hospital stay previous to collection of blood; and no jaundice. The samples of blood were collected in clean, dry tubes, allowed to clot, and centrifuged, and the serum was collected and stored at −25° C. Most of the determinations were done a few days after collection. According to our data, freezing or slight hemolysis does not significantly alter the inhibitory activity of the serum. Duplicate determinations agreed very well. Variations between different batches of substrate were minimized by running both normal sera and sera from cancer patients each day.

RESULTS

The results are tabulated in table 1. Since no correlation was found between either sex or age and inhibitor values in the normal control group of adults, it was assumed that these factors had no appreciable effect on the values obtained in the adult cancer group which was studied. From the table, it is evident that there exists no significant difference between the means of the normal and benign groups. However, there exists a highly significant difference, not only between sera from normal and cancer patients but also between the metastatic and nonmetastatic groups of the latter. Table 2 gives a break-down of the results according to the type of tumor.

DISCUSSION

Friou and Wenner (*23*), using the mucous clot prevention test and hyaluronidase from a species of hemolytic streptococcus, reported relatively high inhibitions for sera of rheumatic fever patients. It seems likely that they were measuring the increase of the specific antibody inhibitor as well. Thompson and Moses (*24*), using the same method, observed an eightfold increase in the inhibiting power of serum in cases of pneumonia when tested against the hyaluronidase from the

TABLE 1.—*Statistical analysis of the mean value of hyaluronidase inhibitor in A/cubic centimeter of normal, benign, and cancerous serum*

Group	Number of cases	Range	Mean	Standard deviation	Deviation from normal	Standard error of deviation	P	Deviation group 4 from group 3	Standard error of deviation	P
1 (normal)	90	6–30	16.2	5.4						
2 (benign)	15	5–36	18.6	8.6	2.4	2.3	0.32			
3 (cancerous, nonmetastatic)	70	10–84	24.1	7.8	7.9	1.1	<.001			
4 (cancerous, metastatic)	80	13–100	38.4	14.7	22.2	1.8	<.001	13.8	1.9	<0.001

bacteria causing the infection. In previous papers, it was demonstrated by means of the same viscosimetric method and materials used in this study that there was an increase in the inhibiting power of blood serum in poliomyelitis (*25*) and certain dermatologic (*26*) and other infections.[5] In the present work, significant elevations were found in human cancer as well. Our studies deal only with the inhibiting power of human serum when tested against hyaluronidase from bull testis. We can merely speculate as to the nature of the hyaluronidase inhibition observed in this work. It seems unlikely that it represents an antibody type of response since one would not expect human serum to contain antibodies to bull testis enzyme. In general it would appear that the nonspecific inhibitor can respond to the stress of certain pathological states and be re-enforced by antibody inhibitors elicited by foreign hyaluronidase protein. The nature of the nonspecific hyaluronidase inhibitor in human serum is now under investigation in our laboratory, and the work on cancer patients and the use of the inhibitor measurement as a possible diagnostic aid in cancer is being continued.

TABLE 2.—*Mean hyaluronidase inhibitor values in A/cubic centimeter serum arranged according to primary site of malignant growth*

Site or type	Nonmetastatic		Metastatic	
	Number	Mean	Number	Mean
Skin and mucous membrane	9	23	2	36
Esophagus			2	41
Stomach	4	24	17	40
Colon and rectum	7	20	6	35
Ovary	2	32	4	58
Cervix	31	26	12	40
Endometrium	7	23	3	35
Breast	7	23	10	31
Prostate	1	20	2	26
Bladder	1	25	3	35
Hypernephroma			1	50
Carcinomatosis			6	38
Parotid	1	13		
Sarcoma			3	33
Melanosarcoma			3	42
Lymphoblastoma			5	39
Multiple myeloma			1	27

[5] GRAIS, M., and GLICK, D.: Unpublished data.

SUMMARY

Inhibition of hyaluronidase activity by blood sera was measured viscosimetrically, hyaluronidase from bovine testes and hyaluronic acid from human umbilical cords being used. Ninety normal sera were assayed and compared with the sera of benign and malignant tumor patients. No significant difference between normal values and those obtained from 15 cases of benign tumors were observed. Sera from 70 cases of nonmetastatic malignant disease showed a mean value which was approximately 52 percent higher than that of the normal group. There was also a still greater increase (140 percent) in the mean value of the 80 cases comprising the metastatic group compared with the normal. All data were treated statistically, and the differences reported were found to be highly significant. The inhibitor is apparently nonspecific in the immunological sense, and the mechanism of its action is still unknown.

ADDENDUM

After this paper was submitted for publication, the communications of Winzler and coworkers, J. Clin. Investigation 27: 609, 617 (1948), appeared in which elevations of plasma mucoprotein levels were reported for patients with cancer and pneumonia. In view of the fact that other investigations, such as those of Seibert et al., J. Clin. Investigation 26: 90 (1947), and Niazi and State, Cancer Research (in press), have also demonstrated that the carbohydrate content of serum proteins is elevated in cancer and infectious diseases, the question arises whether the hyaluronidase inhibitor of the serum actually is, or is associated with, the mucoprotein or carbohydrate compounds studied by these workers. This question is now being investigated in collaboration with Dr. R. J. Winzler, of the University of Southern California Medical School.

REFERENCES

(*1*) Duran-Reynals, F., and Stewart, F.: The action of tumor extracts on the spread of experimental vaccinia of the rabbit. Am. J. Cancer 15: 2790–2796 (1931).

(*2*) Schuerin, Vander G.: L'influence des extraits tumoraux sur les infections virus et staphylococci. Compt. rend. Soc. de. biol. 120: 261–263 (1935).

(*3*) Pirie, A.: A hyaluronidase and a polysaccharide from tumours. Brit. J. Exper. Path. 23: 277–284 (1942).

(*4*) Boyland, E., and McClean, D.: A factor in malignant tissues which increases the permeability of the dermis. J. Path. & Bact. 41: 560–567 (1935).

(*5*) McCutcheon, M., and Coman, D. R.: Spreading factor in human carcinomas. Cancer Research 7: 379–382 (1947).

(*6*) Hoffman, D. C., Parker, F., and Walker, T.: The effect of testicle extract on the Rous sarcoma. Am. J. Path. 7: 523–531 (1931).

(*7*) Duran-Reynals, F.: The effect of testicle extract and of normal serum on a transplantable epithelial tumor of the rabbit. J. Exper. Med. 54: 493–497 (1931).

(*8*) Tanzer, R. C.: The effect of testicle extract on the growth of transplantable mouse tumors. J. Exper. Med. 55: 455–463 (1932).

(*9*) Prime, F., and Haagensen, C. D.: The effect of testicle extract on animal neoplasms. Am. J. Cancer 20: 630–635 (1934).

(*10*) Sturm, E., and Duran-Reynals, F.: Properties of causative agent of chicken tumor; effect of testicle extract on rate of growth of chicken tumor I. J. Exper. Med. 56: 711–717 (1932).

(*11*) Duran-Reynals, F., and Claude, A.: Further experiments on the effect of testicle extract on the agent of chicken tumor I. Proc. Soc. Exper. Biol. & Med. 32: 67–68 (1934).

(*12*) Simpson, W. L., and Gopal-Ayengar, A. R.: Hyaluronidase and the growth of malignant epithelial tumors. Anat. Rec. 97: 369 (1947).

(*13*) Coman, D. R., McCutcheon, M., and Zeidman, I.: Failure of hyaluronidase to increase the invasiveness of neoplasms. Cancer Research 7: 383–385 (1947).

(*14*) Duran-Reynals, F.: The effect of antitesticular serum on the enhancement value of the testicle extract. J. Exper. Med. 55: 703–712 (1932).

(*15*) McClean, D., and Hale, C. W.: The hyaluronidase activity of testicular extracts, bacterial culture filtrates and other agents that increase tissue permeability. Biochem. J. 35: 159–183 (1941).

(*16*) Hobby, G. L., Dawson, M. H., Meyer, K., and Chaffee, E.: The relationship between spreading factor and hyaluronidase. J. Exper. Med. 73: 109–122 (1941).

(*17*) Leonard, S. L., and Kurzrok, R.: A study of hyaluronidase effects on the follicle cells of ovulated rat ova. Endocrinology 37: 171–176 (1945).

(*18*) McClean, D.: The in-vivo decapsulation of streptococci by hyaluronidase. J. Path. & Bact. 54: 284–286 (1942).

(*19*) Haas, E.: On the mechanism of invasion. I. Antinvasin I, an enzyme in the plasma. J. Biol. Chem. 163: 63–88 (1946).

(*20*) Leonard, S. L., and Kurzrok, R. A.: Inhibitors of hyaluronidase in blood sera and their effect on follicle cell dispersal. Endocrinology 39: 85–90 (1946).

(*21*) Hechter, O., and Scully, E.: Studies on spreading factors. II. The effect of serum upon hyaluronidase spreading activity. J. Exper. Med. 86: 19–28 (1947).

(*22*) Hadidian, Z., and Pirie, N. W.: The effects of serum and of hyaluronic acid derivatives on the action of hyaluronidase. Biochem. J. 42: 266–274 (1948).

(*23*) Friou, G. J., and Wenner, H. A.: On the occurrence in human serum of an inhibitory substance to hyaluronidase produced by a strain of hemolytic streptococcus. J. Infect. Dis. 80: 185–193 (1947).

(*24*) Thompson, R. T., and Moses, F. E.: Specificity of human serum anti-hyaluronidase for antagonism of a particular species of bacterial hyaluronidase. Fed. Proc. 7: 282 (1948).

(*25*) Glick, D., and Gollan, F.: Mucolytic enzyme systems. I. Inhibition of hyaluronidase by serum in poliomyelitis. J. Infect. Dis. 83: 200 (1948).

(*26*) Grais, M., and Glick, D.: Mucolytic enzyme systems. II. Inhibition of hyaluronidase by serum in skin disease. J. Invest. Dermat. 11: 259 (1948).

QUANTITATIVE HISTOLOGIC ANALYSIS OF THE EFFECT OF CHRONIC WHOLE-BODY IRRADIATION WITH GAMMA RAYS ON THE SPERMATOGENIC ELEMENTS AND THE INTERSTITIAL TISSUE OF THE TESTES OF MICE[1,2,3]

By ALLEN B. ESCHENBRENNER, ELIZA MILLER, and EGON LORENZ, *National Cancer Institute, National Institutes of Health, Public Health Service*

INTRODUCTION

An earlier publication (*1*) dealt with a quantitative histologic analysis of the effect of chronic whole-body irradiation with X-rays on the interstitial tissue of mouse testes. Mice were exposed to a total dose of 600 r (air) given over a period of 11 weeks at the rate of 8.8 r in 1.65 minutes 6 times per week. The average weight of pairs of testes of 10 mice was 60 mg. as compared with an average weight of 225 mg. for pairs of testes of 10 nonirradiated mice of the same age. It was found that the apparent increase of interstitial tissue in irradiated testes was relative and not absolute so that the decrease of weight was due entirely to a decrease in the quantity of spermatogenic elements. In reviewing these histologic preparations, we gained the impression that there existed apparently normal proportions of spermatogenic elements in various stages of development, despite the fact that their total quantity was only approximately one-fourth of normal.

In order to obtain quantitative data on this problem, the cell populations of the tubules of the testes of mice that have been exposed to whole-body irradiation with gamma rays have been analyzed by means of Chalkley's method (*2*).

A new series of animals exposed to chronic gamma radiation has been used. The cell populations of the tubules of the testes have been analyzed. In addition, the earlier observations (*1*) on the absence of change in quantity of interstitial tissue have been checked and confirmed. Finally, since there is a morphologic change in the male mouse kidney following castration (*3*), the kidneys of some of the mice have been subjected to quantitative histologic analysis to throw some light on the question whether there is a functional change in the interstitial tissue in the absence of the finding of a quantitative one.

EXPERIMENTAL PROCEDURE

The mice were of the same strain as used in the earlier work (*1*), being LAF_1 hybrid males from colonies maintained at the National Cancer Institute. They were 2 months of age at the beginning of irradiation. A detailed description of the irradiation arrangements along with the results of histologic study of organs other than the testes will be given in subsequent papers.[4] For the purpose of this report, a brief outline is sufficient, being the same as that described in another publication dealing with the increase in incidence of lung tumors in mice following long-continued irradiation with gamma rays (*5*). The mice were kept in wooden boxes, eight animals in each. The boxes were made of pine wood 1 cm. thick, with inside dimensions of 28 x 18 x 15 cm. A small food hopper of galvanized iron was placed in the middle of each end board. The top of the boxes consisted of 8-mm.-mesh wire netting nailed to a wooden frame. Drinking water was administered to the mice in wide-mouthed bottles of 100-ml. capacity, closed by a rubber stopper, into which a glass tube approximately 8 mm. in diameter and 10 cm. long was inserted. The bottles were inverted and supported by the wire netting, with the drinking tubes approximately 5 cm. distant from the food hoppers along the midline of the cage. Diet from weaning consisted of Purina dog chow pellets and tap water.

The source of radiation was radium sulphate contained in a capsule of platinum, 40 x 6.4 mm., 0.5 mm. thick. In equilibrium it has an external gamma radiation equivalent of 992.5 mg. of radium element. The geometric center of each box of experimental animals was placed at a distance from the source suitable to obtain a gamma ray intensity of 1.1 r, 4.4 r, or 8.8 r in 8 hours. The distance of the boxes from the source was determined by measurement of the gamma ray intensity

[1] Received for publication March 22, 1948.

[2] This paper is based on work performed under contract number W-31-109-eng-38 for the Manhattan Project at the Argonne National Laboratory.

[3] Presented at the thirty-first annual meeting of the American Society for Experimental Pathology held in Chicago, May 21, 1947. Published in abstract form in Federation Proc. 6 : 391 (1947).

[4] See abstract (*4*).

at the midpoint of the box with Victoreen 0.25 r and 25 r [5] ionization chambers. Because of the varying distance of the different points inside the box from the source, the gamma ray intensity varied from point to point, the maximum variations being approximately ±10 percent. Since the mice were forced to feed and to drink along the midline of the cages, the variations in total doses among different experimental mice was probably somewhat smaller than ±10 percent.

There were three groups of irradiated mice and one group of nonirradiated mice of the same age, the latter being kept in a room distant from that of the irradiated mice but otherwise having the same environmental conditions. The three groups of irradiated mice were exposed, respectively, to 1.1 r, 4.4 r, and 8.8 r gamma radiation given in 8 hours daily. At 2-month intervals from 2 to 16 months after beginning of exposure, four mice from each of the three experimental and the control groups were killed by cervical dislocation. The right testis of each mouse was dissected free of the epididymis and fat and weighed on a torsion balance. It was then fixed in Zenker-formol for 4–5 hours, washed for 12 to 20 hours in running water, and placed in 70 percent ethyl alcohol for several days. Each was then cut into halves. These were dehydrated in ethyl alcohol, and one was embedded in paraffin and the other in celloidin. The sections were cut 7μ in thickness. The celloidin sections were stained with hematoxylin and eosin, and the paraffin sections were stained with Mallory's aniline blue acid fuchsin. The former were used for the study of spermatogenic elements and the latter for study of interstitial tissue. The Mallory's aniline blue acid fuchsin stain is well-suited for the determination of percent of interstitial tissue comprising a section of testis because of the clear differentiation of interstitial tissue cells from stroma and spermatogenic elements.

Quantitative analysis of the testes using Chalkley's method was carried out (*2*). Sections were examined with a 4-mm., 0.95 N. A. apochromat objective (×47.5), and ×15 compensating oculars. One ocular was fitted with four pointers made of fine silver wire, much thinner than the customary mammalian hair pointers. The small diameter of the pointers allowed for rapid and easy determination as to whether or not a pointer touched a particular tissue element. The arrangement of the pointers is shown in figure 1. Their arrangement and number are of no significance other than to suit the convenience of the observer. Data on interstitial tissue and on spermatogenic elements were obtained separately, first to take advantage of the acid fuchsin stain for differentiating interstitial cell cytoplasm while not suitable for identifying spermatogenic elements, and second, to minimize the number of "categories" counted in a given sampling of an organ.

The interstitial tissue was measured as follows: A single section of testis was considered to be representative of the entire organ. A single section of testis of each mouse was scanned by a series of microscope fields chosen at random in progressive series of paths, also chosen at random, in the 2 planes afforded by the mechanical stage of the microscope. In each field the tissue element on which each of the 4 pointers rested was

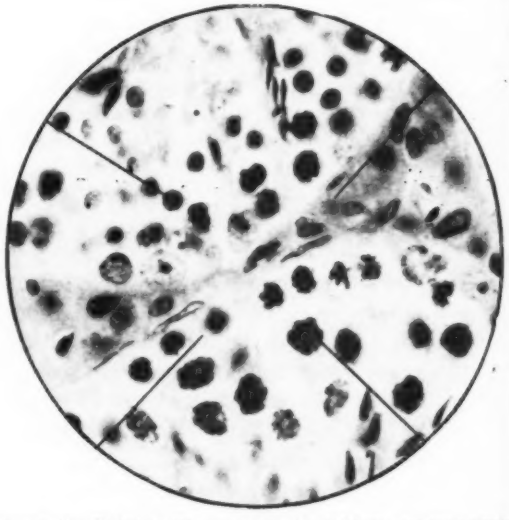

FIGURE 1.—Point pattern as seen in making measurements. The upper left pointer is on a spermatid nucleus; the upper right is on interstitial cell cytoplasm; the lower left is on space; and the lower right is on chromatin of a spermatocyte. The reading is one hit on spermatid nucleus, one hit on interstitial tissue, one hit on space, and one hit on spermatocyte chromatin. From the testis of a mouse that had been exposed to 1.1 r whole-body radiation given in 8 hours daily from 2 to 16 months of age (total dose 540 r). Hematoxylin-eosin stain. ×700.

[5] Calibrated by the National Bureau of Standards with X-rays.

tabulated according to the following classification: (1) Cytoplasm or nucleus of interstitial cells; (2) other tissue; and (3) space (artefacts of thermal and chemical shrinkage). "Hits" on each of the 3 categories of the above classification were recorded with the use of a multiple unit counter having a totalizer. When a total of 100 hits (25 or more microscope fields) had been identified, the numbers of these falling into the 3 categories were recorded. This procedure was repeated 10 times for a total of 1,000 random sampling hits on each section, and the average value was obtained for each category. A sample of such a count was given in an earlier report (*1*) and is not repeated here.

The spermatogenic elements were measured in a similar manner. Celloidin sections 7μ in thickness, stained with hematoxylin and eosin, were used. The tissue element on which each of the 4 pointers rested was recorded according to the following classification: (1) Nuclei of resting spermatogonia; (2) chromatin of spermatogonia in mitosis; (3) chromatin of spermatocytes; (4) nuclei of spermatids; and (5) heads of spermatozoa. Transition stages between the cells of the 5 categories were not counted for reasons brought out later. If a pointer rested on tissue other than the foregoing, or on space (tissue shrinkage), it was not recorded. Tabulations of pointer hits were continued until a total of 100 hits (25 or more microscope fields) was reached, and these were recorded. A series of 10 such tabulations was made on each section, making a total of 1,000 random hits. The sum of each of the 5 categories was obtained and reduced to percent values of each category comprising the total of 5. It is important to point out that this method gives not the relative numbers of cells of the different categories but the relative total area of the nuclei or chromatin of the cells of the different categories. The areas are expressed not as absolute values but as ratios (in percent) of the total area of one category to the sum of the total areas of all categories.

In view of this, the average diameters of the nuclei of spermatogonia and spermatids of the testes of irradiated and nonirradiated mice were measured. In sections of testes of each of 3 mice that had been exposed to 8.8 r daily for 2 months (total dose 1,080 r) and in their respective nonirradiated controls of the same age, the diameters of the nuclei of 30 spermatogonia and 30 spermatids were measured by means of the micrometer eyepiece. The nuclei were chosen at random.

The following criteria were used for inclusion or exclusion of hits on nuclei of spermatogenic elements and for their classification in the five categories used. Nuclei of resting spermatogonia offered no difficulty in identification. Their round or oval nuclei with thin but distinct nuclear margins and their fine to coarse dustlike chromatin were not easily confused with the nuclei of adjacent Sertoli cells or of the interstitial cells of Leydig. Spermatogonia in mitosis and spermatocytes offered some difficulty at first. Our interpretation has been that the chromatin of the spermatogonia in mitosis stains more deeply and has a sharper outline than that of the spermatocytes. The clump of chromatin is likely to be smaller in diameter in the spermatogonia in mitosis and is apt to lie more closely to the basement membrane of the tubule. Also, in both irradiated and normal testes one tends to see groups predominately of spermatogonia in mitosis or of spermatocytes. No attempt was made to differentiate primary and secondary spermatocytes. Spermatid nuclei were not counted when they became noticeably smaller and lost their round shape or the central position of the nucleus in the cytoplasm. Only those spermatozoa heads were counted that were thin, pointed rods that stained intensely with hematoxylin.

The quantitative histologic data on kidneys were obtained by the method of Crabtree, who showed that there is a sex difference in the structure of Bowman's capsule in mouse kidneys (*6*). In intact female and castrated male mice, 20–30 percent of Bowman's capsules are lined partially or completely with cuboidal cells and 70–80 percent are lined completely with squamous cells. In intact males the proportions are the reverse. The structure of the kidney of castrated male mice may be restored to that of intact males by the administration of testosterone (*3*).

In the present experiments, all glomeruli of one section of a kidney were counted and tabulated as having Bowman's capsules lined with squamous cells or lined partially or completely

with cuboidal cells. This counting was facilitated by placing in the microscope ocular an opaque disk having a square opening to restrict the visual field, and by the use of Mallory's aniline blue acid fuchsin stain which prominently stains the basement membrane of Bowman's capsules.

RESULTS

The average weights of the testes of four animals on each irradiation exposure level and of nonirradiated mice of the same age, killed at 2-month intervals, are tabulated in table 1 and shown graphically in figure 2. There was a sharp decrease of testes weights after 2 to 4 months' exposure to 4.4 r and 8.8 r given in 8 hours daily, and thereafter there was no appreciable change of weight, the curves of animals on the two different exposure rates leveling off at distinctly different values. There was a later and slight decrease of weight of the testes of mice exposed at the rate of 1.1 r per day.

The histologic appearance of testes of mice on the three different exposure levels for 16 months and of nonirradiated mice of the same age are shown in figures 3 and 4. In contrast, figure 5 shows the appearance of testes of mice exposed to the same total accumulated dose (2,160 r) but at different exposure rates (8.8 r and 4.4 r given in 8 hours daily). The changes that were observed in relation to rate of irradiation and in relation to duration of irradiation are described separately under considerations of interstitial tissue and spermatogenic elements of the testes.

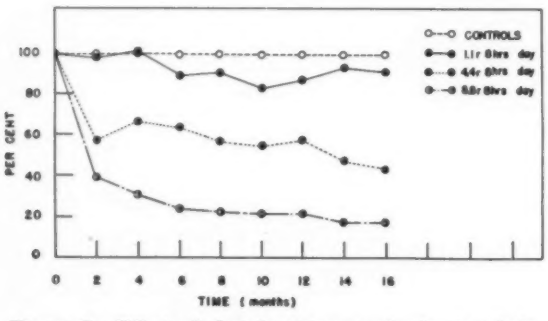

FIGURE 2.—Effect of chronic exposure of mice to whole-body gamma radiation at rates of 1.1 r, 4.4 r, and 8.8 r given in 8 hours daily as reflected in weights of testes. Each point on the curves is an average value for 4 mice. Testes weights of irradiated mice are expressed as percent of weight of testes of nonirradiated mice of the same age.

The data in table 1 show that with decrease in weights of testes as a result of chronic irradiation, the proportion of the testes composed of interstitial tissue increased. This is shown in a striking manner by the data on animals killed at 18 months of age. In nonirradiated animals the interstitial tissue comprises an average of 6.4 percent of the total volume of the organ. In animals exposed to a total dose of 2,160 r at the rate of 4.4 r given in 8 hours daily, it comprises 12.4 percent; and in animals exposed to a total dose of 4,320 r at the rate of 8.8 r given in 8 hours daily, it comprises 27.7 percent of the total volume of the testes. That this increase of interstitial tissue is relative and not real is indicated by multiplication of these percent values by the respective testes weights. This calculation gives a theoretical value for total quantity of interstitial tissue per testis. Table 1 shows that the total quantity of interstitial tissue per testis remains essentially unchanged.

TABLE 1.—*Data on mice exposed daily to long-continued whole-body gamma radiation* [1]

Experimental condition	Age	Period of irradiation	Total dose (r)	Body weight	Testis weight	Interstitial tissue of testis	
	Months	*Months*		*Grams*	*Milligrams*	*Percent*	*Milligrams*
Nonirradiated controls	2	0	0	24.0	96	5.26	5.00
	4	0	0	30.5	113	4.62	5.22
	6	0	0	35.1	114	5.56	6.40
	8	0	0	36.1	118	5.86	6.90
	10	0	0	37.2	111	6.33	7.06
	12	0	0	34.8	110	5.75	6.29
	14	0	0	37.2	115	6.29	7.10
	16	0	0	36.1	115	6.59	7.57
	18	0	0	40.4	119	6.40	7.68
1.1 r given in 8 hours daily	4	2	68	32.0	112	4.30	4.85
	6	4	135	39.9	116	5.86	6.81
	8	6	203	43.9	108	5.01	5.42
	10	8	270	42.4	101	6.16	6.40
	12	10	338	49.6	99	7.85	6.88
	14	12	406	43.0	102	6.53	6.68
	16	14	474	40.7	109	7.38	7.97
	18	16	540	36.4	110	6.12	6.71
4.4 r given in 8 hours daily	4	2	270	31.7	64	8.00	5.06
	6	4	540	39.4	76	6.71	5.15
	8	6	810	43.4	77	7.40	5.67
	10	8	1,080	39.8	64	10.50	6.22
	12	10	1,350	40.3	65	8.24	5.37
	14	12	1,620	40.5	69	8.65	5.95
	16	14	1,890	33.6	56	10.24	5.70
	18	16	2,160	33.7	52	12.40	5.64
8.8 r given in 8 hours daily	4	2	540	31.0	44	11.9	5.22
	6	4	1,080	38.1	36	20.7	5.96
	8	6	1,670	40.7	28	24.5	6.82
	10	8	2,160	43.7	26	29.2	7.46
	12	10	2,700	34.5	26	29.3	7.71
	14	12	3,240	41.5	25	26.2	6.70
	16	14	3,780	32.4	22	24.1	5.26
	18	16	4,320	31.1	22	27.7	6.10

[1] Each weight represents an average value for 4 mice. Weight of interstitial tissue is a theoretical value obtained by multiplying the fresh testis weights by the percent value for interstitial tissue obtained histologically by Chalkley's method.

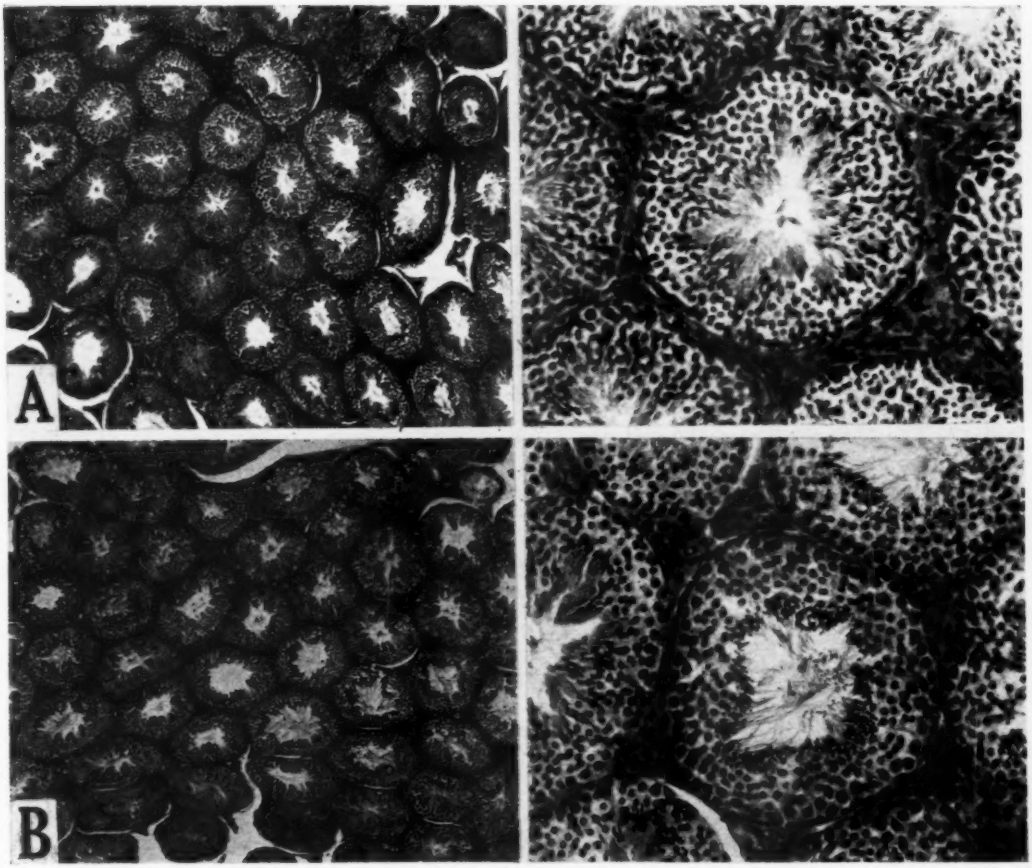

FIGURE 3.—*A*, Testis of nonirradiated mouse 18 months of age. × 50 and × 200. *B*, Testis of mouse 18 months of age that had been exposed to 1.1 r whole-body gamma radiation given in 8 hours daily beginning at 2 months of age (total dose 540 r). × 50 and × 200.

The results of measurement of diameters of nuclei of spermatogonia and spermatids in sections of testes of irradiated and nonirradiated mice are given in table 2. There is no appreciable difference in measurements made on the treated and untreated mice. The frequency distribution of nuclear sizes in the two groups of animals was analyzed by the 2 x n χ^2 technique. The value obtained was $\chi^2=0.04528$. The probability of the frequency distribution being different was less than one in one hundred.[6]

The results of determination of cell populations in the tubules of the testes are given in table 3. These determinations were not made on the testes

[6] The statistical analysis was made by D. George Deihl.

TABLE 2.—*Average diameter of nuclei of spermatogonia and of spermatids in testes of irradiated and nonirradiated mice as measured with micrometer eyepiece*[1]

Condition	Average diameter of spermatogonia nuclei	Average diameter of spermatid nuclei
	Microns	*Microns*
Nonirradiated	5.9	5.5
	5.4	5.8
	5.5	5.2
Average	5.6	5.5
Irradiated	5.5	5.5
	5.8	5.3
	6.1	5.9
Average	5.8	5.6

[1] Each value is an average for 30 nuclei chosen at random. The irradiated mice were exposed to 8.8 r whole-body gamma radiation given in 8 hours daily for 2 months (total dose 540 r).

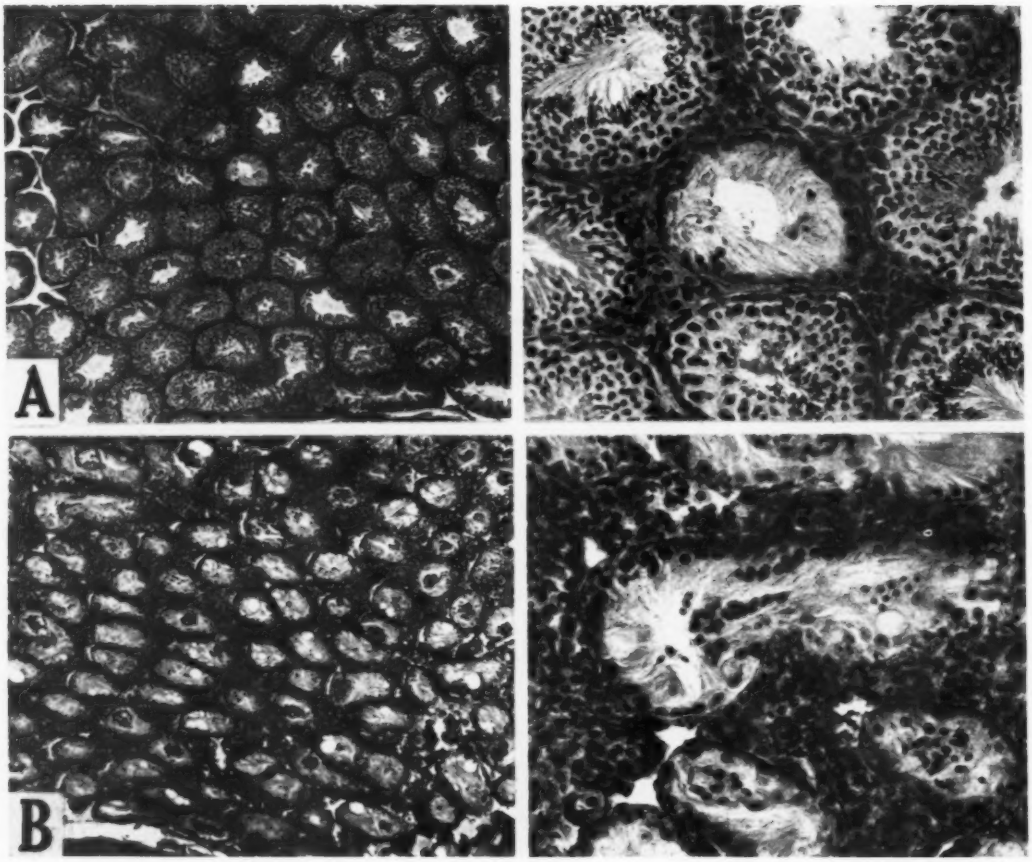

FIGURE 4.—*A*, Testis of mouse 18 months of age that had been exposed to 4.4 r whole-body gamma radiation given in 8 hours daily beginning at 2 months of age (total dose 2,160 r). Note that all but two tubules appear normal. One of the abnormal tubules shown in higher magnification is seen to contain mostly Sertoli cells and in addition a few spermatogonia and spermatozoa. × 50 and × 200. *B*, Testis of a mouse 18 months of age that had been exposed to 8.8 r whole-body gamma radiation daily beginning at 2 months of age (total dose 4,320 r). No normal tubules are seen, and only one of the tubules contains numerous spermatogenic elements (shown in higher magnification). × 50 and × 200.

of all animals. In the case of animals exposed to 1.1 r and to 4.4 r given in 8 hours daily, no significant differences were found in the relative values for elements in different stages of spermatogenesis after irradiation for 16 months, as compared with these values in nonirradiated mice of the same age. No determinations were made, therefore, on testes of animals exposed for shorter periods. In the case of animals exposed to 8.8 r given in 8 hours daily, determinations were made only in mice irradiated for 2 to 4 months, and these showed no differences as compared with other mice. After periods of irradiation longer than 4 months, a random sampling could not be made because of irregularity of location of tubules containing spermatogenic elements. As shown in figures 5, 6, and 7, the occurrence of relatively large numbers of spermatogenic elements in a few tubules precludes such a random sampling. An attempt was made to arrive at quantitative data on these testes by counting each spermatogenic element in each tubule. A trial was made of this method.

TABLE 3.—*Analysis of the cell populations of the seminiferous tubules of the testes*[1]

	Age	Time irradiated	Total dose (r)	Spermatogenic elements: relative total areas of random samples of nuclear material in histologic sections				
				Spermatogonia resting	Spermatogonia in mitosis	Spermatocytes	Spermatid	Spermatozoa heads
	Months	*Months*		*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Nonirradiated controls	4	0	0	12.1	14.1	28.1	41.9	3.8
	4	0	0	9.0	10.5	29.7	45.4	5.4
	4	0	0	8.3	11.1	33.5	43.1	4.0
	4	0	0	6.4	8.6	36.5	40.3	4.2
Average				8.9	11.1	31.9	43.6	4.3
	6	0	0	6.4	5.5	40.6	43.0	4.5
	6	0	0	10.7	3.0	42.2	38.6	5.5
	6	0	0	9.8	2.8	39.4	43.2	4.8
	6	0	0	7.6	6.0	35.6	44.2	6.6
Average				8.6	4.3	39.4	42.2	5.3
	18	0	0	6.4	6.0	38.4	43.6	5.6
	18	0	0	6.2	6.0	39.4	43.2	5.2
	18	0	0	8.8	4.0	35.6	46.2	5.4
	18	0	0	8.6	7.0	45.6	34.4	4.4
Average				7.5	5.7	39.7	41.8	5.1
1.1 r given in 8 hours per day	18	16	540	8.0	2.0	38.2	46.6	5.2
	18	16	540	10.6	3.4	35.6	46.8	3.6
	18	16	540	10.8	2.2	35.2	46.0	5.8
	18	16	540	10.6	3.6	45.0	37.6	3.2
Average				10.0	2.8	38.5	44.2	4.4
4.4 r given in 8 hours per day	18	16	2,160	15.0	6.2	38.0	38.2	2.6
	18	16	2,160	6.6	7.8	40.2	40.6	4.8
	18	16	2,160	14.8	5.8	35.8	36.8	6.8
	18	16	2,160	9.6	5.6	36.8	41.6	6.4
Average				11.5	6.3	37.7	39.3	5.1
8.8 r given in 8 hours per day	4	2	540	8.8	10.3	35.3	40.9	4.7
	4	2	540	7.0	4.9	32.9	50.6	4.6
	4	2	540	7.0	11.7	32.7	44.7	3.9
	4	2	540	7.7	6.6	35.3	47.8	2.6
Average				7.6	8.37	34.0	46.0	3.95
	6	4	1,080	9.0	5.6	39.0	44.0	2.4
	6	4	1,080	8.8	4.2	39.4	43.0	4.6
	6	4	1,080	7.6	2.6	41.0	45.0	3.8
Average				8.46	4.1	39.8	44.0	3.6

[1] Results of application of Chalkley's method to an analysis of the cell populations of the seminiferous tubules of the testes of mice exposed daily to whole-body gamma radiation, and of nonirradiated mice of the same ages. Each value is that for a single mouse.

Since the identification and recording of all elements in less than half the tubules of one section by one of us consumed 4 hours, this method was discarded.

Observations on the testes other than the quantitative data were as follows: In mice exposed to 1.1 r given in 8 hours daily and killed at intervals of 2 months after periods of 2 to 16 months of irradiation, sections of testes could not be distinguished from those of nonirradiated mice of the same age. The tubules appeared to be of the same average diameter as those of the controls (fig. 3, *A* and *B*). There was no difference in the contents of the tubules. In animals exposed to 4.4 r given in 8 hours daily, however, there were usually one or more tubules in a section that were nearly devoid of spermatogenic elements (fig. 4, *A*). Furthermore, the average diameter of tubules appeared to be less in the animals exposed to 4.4 r daily. This will be apparent by comparing figures 3, *A* and 4, *A*, photomicrographs taken in succession under the same optical conditions.

The greater importance of dose rate as compared to total dose with regard to the changes produced in the testes by whole-body irradiation are strikingly shown in figure 5. The two photomicrographs are from animals that had received the same total dose of irradiation, one receiving this at the rate of 4.4 r given in 8 hours daily, and the other at the rate of 8.8 r given in 8 hours daily. These photomicrographs also strikingly point out an apparent interstitial cell hyperplasia which

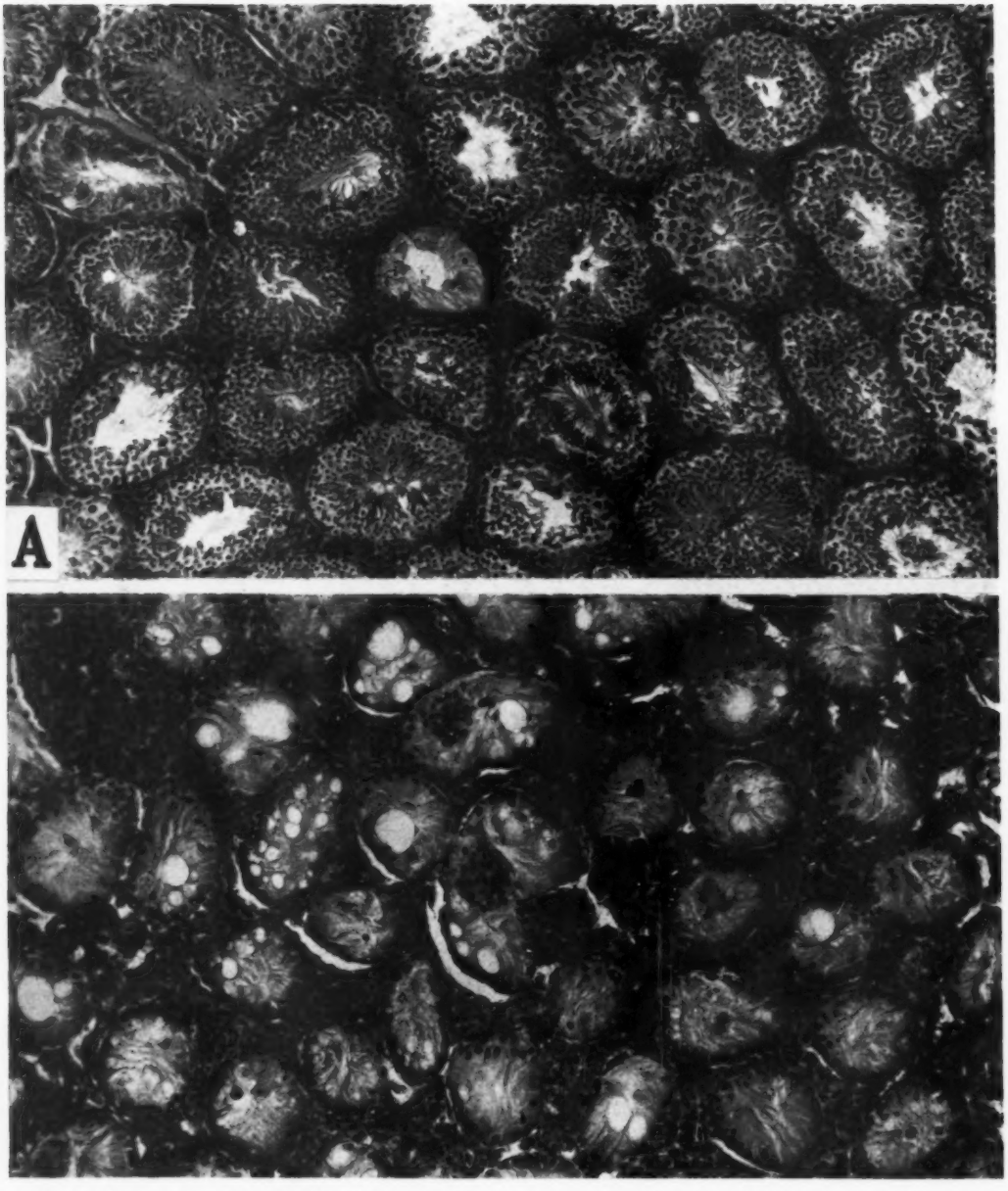

FIGURE 5.—Sections of testes of mice that had been exposed to the same total dose (2,160 r) but at different rates. *A*, Section of the testis of a mouse that had been exposed to 4.4 r given in 8 hours daily, from 2 to 16 months of age. × 120. *B* (lower figure), Sections of testis of a mouse that had been exposed to 8.8 r given in 8 hours daily from 2 to 8 months of age. × 120. Note the striking difference in degree of damage to spermatogenic elements and in the relative (although not real) increase of interstitial tissue.

has been shown by means of Chalkley's method not to be real.

A striking difference was noted between the testes of mice exposed to 8.8 r given in 8 hours daily and the testes of nonirradiated mice or those exposed to 4.4 r or to 1.1 r daily. After irradiation for 2 and for 4 months, the quantitative analyses revealed no change in proportion of spermatogenic elements in different stages of development. Although no quantitative analyses of this type could be made in animals irradiated for 6 to 16 months, distinct qualitative changes were noted. The occurrence of apparently normal spermatogenesis in a few tubules and its almost complete absence in remaining tubules after 6 months has been noted (fig. 5, *A*). After 10 months of irradiation, spermatozoa were relatively few in number, as compared with less mature spermatogenic elements (figs. 5, 6, and 7). Furthermore, multinucleated spermatids were common (figs. 5, lower figure; 6, *B*; 8, *B*, and 8, *D*). Finally, what is probably a most significant finding in relation to the foregoing was the occurrence of degeneration of Sertoli cells (fig. 8, *B* and *C*).

The degenerating Sertoli cells occurred in masses, usually in the center of the lumina of the seminiferous tubules. Occasionally the syncytial masses of cytoplasm containing the abnormal nuclei were attached to the cytoplasm of the normal nuclei at the periphery of the tubule, and a series

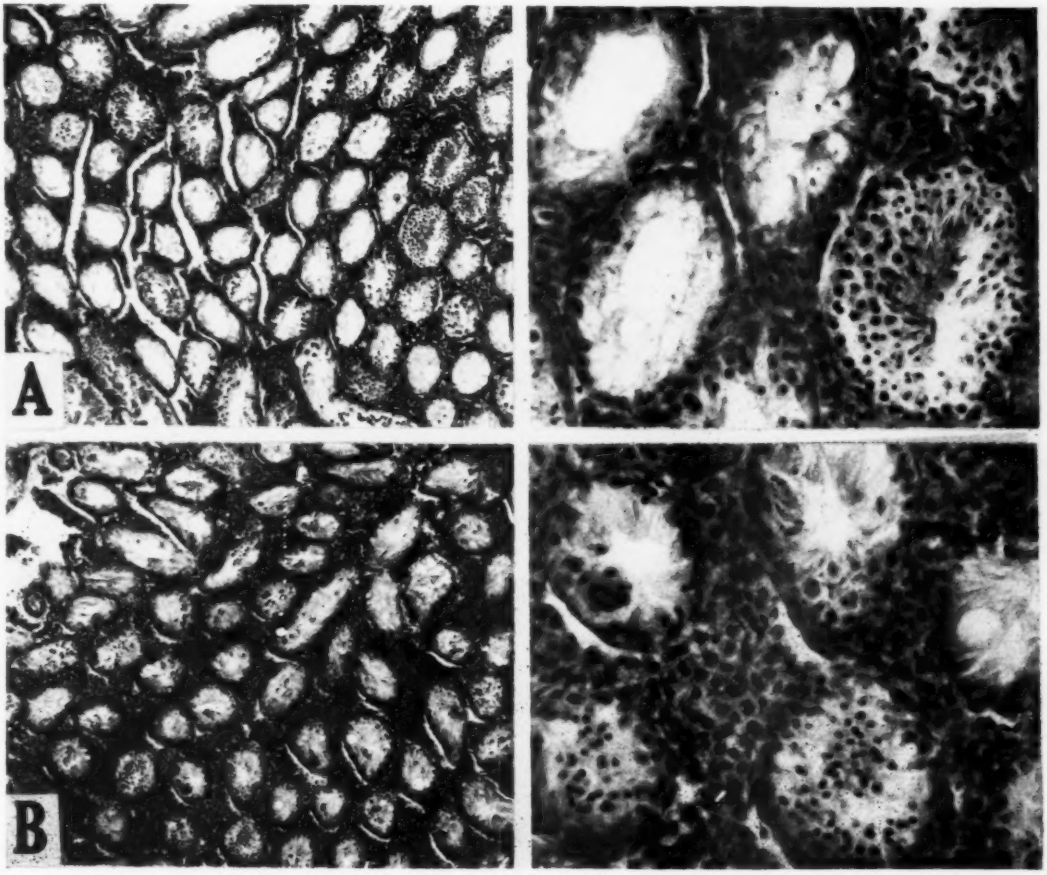

FIGURE 6.—Sections of testes of mice that had been exposed to 8.8 r whole-body gamma radiation in 8 hours daily for 6 months, *A*, and for 10 months, *B*. In A note the completeness of spermatogenesis in some tubules and the absence of it in others. In *B*, spermatogenesis is incomplete, and there are abnormal spermatids (multinucleated). × 50 and × 200, respectively.

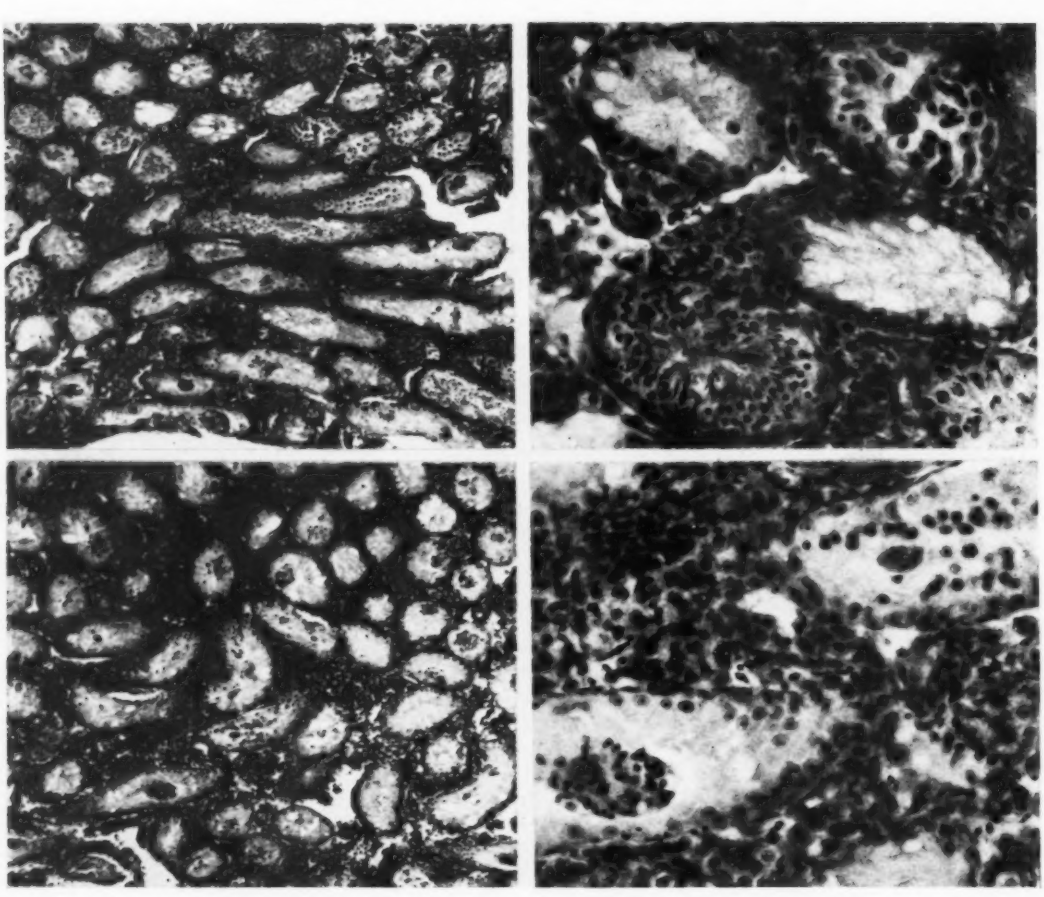

FIGURE 7.—Sections of testes of mice that had been exposed to 8.8 r whole-body gamma radiation given in 8 hours daily for 12 months. In upper figures, note the occurrence of normal and complete spermatogenesis in some tubules, of incomplete spermatogenesis with abnormal spermatids in others, while most tubules are nearly devoid of spermatogenic elements. In lower figures, note the abnormal spermatids (multinucleated), and masses of degenerating Sertoli cells. × 50 and × 200, respectively.

of nuclei in progressive stages of shrinkage could be seen in a given area (fig. 8, *C*). These degenerating nuclei contained the prominent acidophilic and eosinophilic components of the nucleolus complex characteristic of Sertoli cells. Their fate is indicated by the finding of transition stages between the shrunken nuclei, pyknotic nuclei, and finally amorphous deeply basophilic material resulting from fragmentation. In view of this continued degeneration of Sertoli cells, we have looked for evidence of their mode of replacement. New Sertoli cells appear in these sections to be arising through differentiation of stromal elements. More detailed study will be required before this can be definitely established. This problem is now being investigated and will be the subject of a future publication.

The stroma of the testes of the chronically irradiated mice showed no significant changes. The capsule became thickened but remained relatively acellular. The degree of thickening appeared to be proportional to the decrease in size of the organ. The reticular fibers forming the basement membranes of the seminiferous tubules also became slightly more prominent in sections stained with Mallory's aniline blue. Again, this thicken-

ing seemed to be proportional to the decrease in diameter of the tubules. No vascular changes or leukocytic infiltration were observed.

The degrees of suppression of spermatogenic activity in mice on the different exposure levels were reflected in the contents of the epididymides. The epididymides of animals exposed to 1.1 r given in 8 hours daily could not be distinguished from those of the nonirradiated controls. In mice exposed to 4.4 r given in 8 hours daily there was a noticeable decrease in the number of spermatozoa in the tubules of the epididymides. No abnormal elements were present. On the other hand, after 6 months or more of irradiation at the rate of 8.8 r given in 8 hours daily, the lumina of the epididymides contained no cellular elements or debris. In other respects they were normal. After 2 months of irradiation on this level, there was a decrease of spermatozoa in the epididymides of two of four mice, and after 4 months of irradiation the spermatozoa were absent in two and rare in two mice.

The results of classification of glomeruli in the kidneys of mice that had been exposed to 8.8 r, 4.4 r, and to 1.1 r whole-body radiation given in 8 hours daily, and of nonirradiated male mice of the same age are shown in table 4. Since the values for the proportions of the two types of

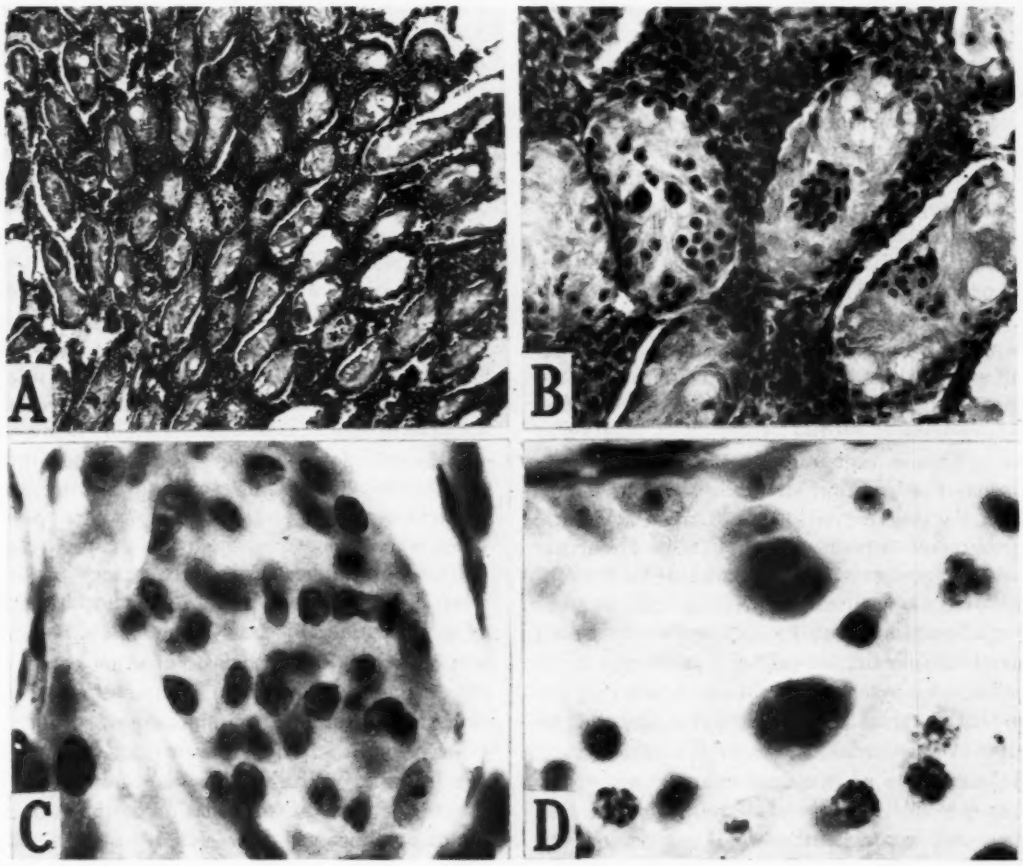

FIGURE 8.—Sections of testis of a mouse that had been exposed to 8.8 r whole-body gamma radiation given in 8 hours daily from 12 to 16 months of age (total dose 3,780 r). Note the scarcity of spermatogenic elements (*A*); the presence of degenerating and multinucleated spermatids (*B* and *D*); and degenerating Sertoli (*B* and *C*). *A*, × 50; B, × 200; C and D, × 800.

TABLE 4.—*Results of analyses of kidney for percent of Bowman's capsules lined with squamous cells and with cuboidal cells* [1]

Rate of chronic daily irradiation	Total dose (r)	Lining of Bowman's capsules of kidneys	
		Squamous	Cuboidal
		Percent	*Percent*
Nonirradiated		19.6	80.4
1.1 r in 8 hours daily	540	29.9	70.1
4.4 r in 8 hours daily	2,160	24.1	75.9
8.8 r in 8 hours daily	4,320	29.7	70.3

[1] Each value represents an average for 4 mice. All mice were 18 months of age at time of autopsy. Irradiation was given daily from 2 to 18 months of age.

glomeruli in castrated mice are the reverse of normal males (*3*), the slight differences shown in table 3 are probably not significant.

DISCUSSION

The testes weight curves shown in figure 1 are of interest for theoretical and practical reasons. The testes weights of mice exposed to 8.8 r per day declined rapidly at first and then leveled off at a value of 20–25 percent of normal. Histologic examination of these testes showed a rapid decrease of spermatogenic elements followed by almost complete loss of these elements after 6 or more months of irradiation, obviating a further decrease of weight. This was not entirely true, however, of animals exposed to 4.4 r per day. Their testes weights showed a rapid decline and a leveling off at a value of 50–60 percent of normal. Histologic examination of these testes revealed active spermatogensis in all but an occasional tubule of section. This is interpreted to indicate that after 4 months of irradiation an equilibrium is reached between the destructive effects of irradiation and the generative capacity of this tissue. Further studies are necessary to obtain any insight into the nature of this equilibrium, whether it is a reflection of the acquisition of slight resistance by all elements, of selection of cells for resistance to irradiation, of a reserve generative capacity of the tissue that requires a latent period to develop, or whether extragonadal factors are involved.

The curves are of practical value in setting up experiments for comparative radiation studies. The present experiments show the testes to be good test organs for quantative evaluation of the biologic effects of radiation. In homogeneous inbred strains of mice, there is little variation in normal testes weights or in the decrease of testes weights resulting from a given irradiation condition. If the testes are used as test organs in experiments designed to measure the effect of accessory factors during chronic irradiation, a daily dose of more than 1 r and less than 8 r should be used if the experiments are to exceed 4 months' duration. If higher daily doses are used, the experiments should be terminated before the testes have been depleted of spermatogenic elements. This information has recently been applied to advantage in a series of experiments designed by Lorenz to study the intensity factor in chronic irradiation (*7*).

The rationale of the method used for measurement of relative and absolute amounts of interstitial tissue has been given in a recent publication (*1*) and need not be reiterated. A comparison of figures 3, *A*, and 4, *B*, and 7, *A* and *B*, would lead one to believe that hyperplasia of the interstitial tissue occurred in the testes of the irradiated animal. In sections of normal testes the interstitial tissue comprises 6–7 percent of a microscope field, while in section of testis of mice exposed daily to 8.8 r radiation it comprises 26–29 percent of a microscope field. That this is only a relative increase and not an absolute one is indicated by multiplication of the percent values by the respective testes weights (table 1).

As pointed out previously (*1*), this study emphasizes a fact that is generally understood but commonly left out of consideration: How much we depend upon relative rather than absolute values in viewing sections through the microscope. With the exception of the use of a comparison eyepiece fitted to two microscopes, in comparing sections we depend upon visual memory in passing from one slide to the next. We compare the size of a particular object with the size of the microscope field and with the size of other components of a section. The former comparison is complicated if we change from one magnification to another when a small portion of a section arouses our interest. With a lack of absolute values in such a comparison, anticipation of the fulfillment of our preconceived theories may have a distorting influence on our final conclusions. These hazards of microscopy can be eliminated by the use of Chalkley's method. A wider application of this

method offers a partial solution to sorely needed quantitative methods in histology and pathology.

The determinations of the relative numbers of the two types of Bowman's capsules in the kidneys probably would not show increased hormone output of interstitial tissue but would show lessened output. The slight differences found between the kidneys of nonirradiated and those of mice exposed to different daily amounts of radiation (table 4) are not considered to be significant. This adds to other evidence that the absence of spermatogenic elements is not a contributing factor of any importance in producing the changes observed in castrated mice. These views are in accord with those of Hu and Frazier (*8*) but not with those of Witschi, Levine, and Hill (*9*). However, both groups of workers studied the effects of acute rather than chronic irradiation, and of irradiation to the testes only and not whole-body irradiation, so that a detailed discussion of their work is not warranted. Further suggestive evidence in this connection could be obtained by comparing the susceptibility of irradiated and nonirradiated mice to renal necrosis produced by chloroform since it has been found that intact males and testosterone-treated castrated males are susceptible while castrated males and intact females are not (*10*).

The method used for the determination of cell population in the spermatogenic tubules of the testes represents a new application of Chalkley's method. With this method one obtains not relative cell numbers, but relative areas of nuclei or chromatin of cells in different stages of spermatogenesis. That it is a reflection of cell numbers is indicated by the observations that the nuclear diameters of spermatogonia and of spermatids do not differ in nonirradiated mice and in mice exposed to 540 r whole-body irradiation over a period of 2 months. Casual observation of sections of normal testes shows considerable variation in the content of spermatogenic elements in different tubules. Such variation is even more striking in the testes of irradiated mice. As a random sampling method for rapidly determining the average relative numbers of cells in different stages of spermatogenesis in all tubules of a given section of testis, Chalkley's method has no equal. An alternative method would be the identification and tabulation of every element in every tubule in a section, but this process would be so tedious and so time consuming as to be discarded at once by most investigators.

Before the results of analyses of spermatogenic elements are discussed, it is in order to consider the stages of spermatogenesis that were chosen for measurement. These consisted of nuclei of resting spermatogonia, chromatin of spermatogonia in mitosis, chromatin of spermatocytes, nuclei of spermatids, and heads of mature spermatozoa. Cytoplasm of these cells was excluded because of its poor definition. Excepting spermatogonia in mitosis, all other transition stages were excluded because their inclusion would have doubled the number of categories counted, requiring more than twice the time to make determinations of equal accuracy, and enumeration of the transition stages would have added little useful information. Separate enumeration of primary and secondary spermatocytes would have introduced considerable difficulty in identification and was not undertaken after preliminary measurements revealed no change in proportions of their progeny, the spermatids.

The results of analyses of spermatogenic elements clearly show a normal proportion of all stages in mice exposed chronically at the rate of 1.1 r and 4.4 r given in 8 hours daily. The same results were obtained at the rate of 8.8 r given in 8 hours daily for as long as 4 months (total accumulated dose being 1,080 r). After this time, spermatozoa were obviously decreased or absent and abnormal spermatids and Sertoli cells were present.

After mice had been exposed to 4.4 r daily for 16 months, their average testes weight was 44 percent of that of the testes of nonirradiated mice of the same age. From the measurements of interstitial tissue, it can be concluded that this means a reduction of quantity of spermatogenic elements to approximately 44 percent of normal, yet the proportion of these elements in different stages of spermatogenesis was the same as normal. The same was true of mice exposed to 8.8 r per day for 4 months. In these, the average testes weight was 32 percent of nonirradiated mice the same age.

The importance of dose rate is shown by the

difference in weights of testes of mice exposed to 8.8 r and 4.4 r in 8 hours daily. On these exposure levels, the accumulated dose was 2,160 r after 8 months of exposure to 8.8 r given in 8 hours daily and after 16 months of exposure to 4.4 r given in 8 hours daily. The respective testes weights were 26 mg. for the 8.8 r dose rate and 52 mg. for the 4.4 r dose rate. The effect of chronic irradiation on the testes, therefore, seems largely if not entirely to be dependent upon dose rate rather than upon total accumulated dose. This confirms previous observations (*1*, *4*). It is in striking contrast, however, with the effect of chronic irradiation on ovaries wherein a given degree of pathologic change is not a function of dose rate or total dose but is dependent upon a minimum dose (*4*). From these results, two alternative conclusions can be drawn regarding the effect of whole-body gamma radiation on the spermatogenic elements. One can conclude that all stages of spermatogenesis have equal sensitivity to irradiation. This might be a reflection of an equal degree of retardation of development of all stages. On the other hand, it could not be a reflection of the destruction of equal numbers in all stages, because of the reduction divisions resulting in doubling of numbers of progeny. A second conclusion that appears to be more logical is that chronic irradiation results in a retardation of the rate of multiplication of spermatogonia. That the decrease in total numbers of spermatogonia is not attributable to a lethal effect on these cells is indicated by the absence of degenerating forms. In this connection it is interesting that in mice exposed to 8.8 r daily for 12 to 16 months unusually large but otherwise normal spermatogonia nuclei are seen (fig. 5, *C*). These might be interpreted as nuclei that have grown in size preparatory to division but have been prevented from dividing.

Aside from the results of the quantitative analyses, the most interesting finding in these experiments is the presence of degenerating Sertoli cells and, associated with this phenomenon, the decrease of spermatids. Testes of all mice exposed for 8 hours daily to 8.8 r for 6 months or longer showed these changes. The authors' present opinion is that the decrease of spermatozoa and the degeneration of spermatids is not due directly to irradiation but only indirectly through degeneration of Sertoli cells. On the other hand, it should be pointed out that such abnormal spermatids are seen in cryptorchid testes. Since irradiated testes are smaller than normal, they may tend to reside more frequently than normal in the abdominal cavity, so that here there might be a combination of irradiation effects and hyperthermal effects. However, degeneration of Sertoli cells of a type and degree seen in these testes is not characteristic of cryptorchid testes. That cryptorchidism is a factor in the production of these changes appears unlikely to the authors.

If our interpretation is correct, that Sertoli cells are arising through differentiation of stromal elements to replace those that are degenerating, it would establish an answer to a controversial question. In a normal testis, replacement of Sertoli cells takes place at such a slow rate that their origin has not been established with certainty. The fact that there is a degeneration of Sertoli cells taking place in the testes of irradiated mice and that they are replaced offers a means of establishing their origin. As mentioned, it appears to us at present that they arise through differentiation of stromal elements. This problem is being investigated and will be the subject of a future communication.

SUMMARY

LAF_1 male mice were exposed to 8.8 r, 4.4 r, or 1.1 r, respectively, whole-body gamma radiation given in 8 hours daily beginning at 2 months of age. Four mice on each dose level and four nonirradiated mice of the same age were killed at intervals of 2 months after 2 to 16 months of irradiation. Only the findings in the testes of these mice are reported. Data include fresh testes weights, quantitative histologic analyses of the relative and absolute amounts of interstitial tissue per testis and of the cells in different stages of spermatogenesis in the seminiferous tubules, and analyses of the morphology of Bowman's capsules in the kidneys as an index of hormone output of the testes.

In mice exposed to 4.4 r and 8.8 r daily there was a sharp decrease of testes weights after 2

and 4 months of irradiation. Thereafter the weight curves leveled off at different values for the different dose rates.

The relative amount of interstitial tissue increased in proportion to decrease of testes weights. Quantitative histologic analysis showed this relative increase was not an absolute one, the quantity of interstitial tissue per testis remaining the same as that of the testes of nonirradiated mice. Analysis of the morphology of the kidneys revealed no evidence of measurable decrease of hormone output of the testes of irradiated mice.

Despite marked decrease of total quantity of spermatogenic elements in the testes of irradiated mice, the proportion of these cells in different stages of spermatogenesis was normal in mice exposed to 1.1 r and 4.4 r daily for as long as 16 months. In mice exposed to 8.8 r daily, it was normal for 4 months, while after 6 or more months of irradiation, there was failure of completion of spermatogenesis, and this was associated with degeneration of Sertoli cells.

Some theoretical implications of the data are discussed, and the practical value of the mouse testis as a test object in quantitative experiments on the biologic effect of radiation is indicated.

REFERENCES

(1) ESCHENBRENNER, A. B., and MILLER, E.: Quantitative histologic analysis of the effect of radiation on the interstitial tissue of the testes of LAF_1 mice. J. Nat. Cancer Inst. 6: 343–348 (1946).

(2) CHALKLEY, H. W.: Method for the quantitative morphologic analysis of tissues. J. Nat. Cancer Inst. 4: 47–53 (1943).

(3) CRABTREE, C.: The structure of Bowman's capsule in castrate and testosterone-treated male mice as an index of hormonal effects on the renal cortex. Endocrinology 29: 197–203 (1941).

(4) LORENZ, E., HESTON, W. E., ESCHENBRENNER, A. B., and DERINGER, M. K.: Biological studies in the tolerance range. Radiology 49: 274–285 (1947).

(5) LORENZ, E., HESTON, W. E., DERINGER, M. K., and ESCHENBRENNER, A. B.: Increase in incidence of lung tumors in strain A mice following long-continued irradiation with gamma rays. J. Nat. Cancer Inst. 6: 349–353 (1946).

(6) CRABTREE, C.: Sex differences in the structure of Bowman's capsule in the mouse. Science 91: 299 (1940).

(7) LORENZ, E., and ESCHENBRENNER, A. B.: The role of intensity in chronic irradiation with gamma and X-rays. Fed. Proc. 6: 395–396 (1947).

(8) HU, C. K., and FRAZIER, C. N.: Atrophy of germinal epithelium of the rabbit's testis induced by roentgen rays. Proc. Soc. Exper. Biol. & Med. 48: 44–48 (1941).

(9) WITSCHI, E., LEVINE, W. T., and HILL, R. T.: Endocrine reactions of X-ray sterilized males. Proc. Soc. Exper. Biol. & Med. 29: 1024–1026 (1932).

(10) ESCHENBRENNER, A. B., and MILLER, E.: Sex differences in kidney morphology and chloroform necrosis. Science 102: 302–303 (1945).

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DISTRIBUTION OF N-ACETYL-2-AMINOFLUORENE IN THE RAT FOLLOWING A SINGLE FEEDING[1,2]

By HAROLD P. MORRIS and BENTON B. WESTFALL, *National Cancer Institute, National Institutes of Health, Public Health Service*

INTRODUCTION

The development of a rapid, sensitive, and reproducible procedure (*1*, *2*) for detection of N-acetyl-2-aminofluorene [3] in tissues and body fluids provided one means for studying the distribution and metabolism of this carcinogen. AAF, the compound administered, is rapidly converted in the organism to some as yet unidentified complexes which were extracted from the tissues by acetone, and, after hydrochloric acid hydrolysis, were analyzed for aminofluorene.[4] Although frequent reference is made to AAF, it should be kept clearly in mind that AAF, as such, was not estimated. Because the method of estimating AAF in living tissue was sensitive at concentrations known to be carcinogenic, it was possible to study some of the problems involved in the mechanisms of carcinogenesis, i. e., the occurrence of the agent in a given tissue, the concentration of the added agent in such tissue following graded dosage, and the correlation of these findings with subsequent tumor incidence.

Study of the metabolism of carcinogenic compounds is not new, yet little progress has been made in understanding their metabolism mainly because the methods of detection have been inadequate to trace the minute quantities of carcinogen needed to induce neoplasms. Furthermore, it was recognized that the metabolism and action of massive doses of a carcinogen may differ from that of doses just enough to induce tumors. This disadvantage inherent in some of the early investigations of carcinogen metabolism has been obviated in these experiments by the application of a suitably sensitive analytical procedure, and at the same time it has been possible to study the distribution and concentration of AAF at different carcinogenic levels of administration.

Many of the known carcinogenic compounds are active only at or near the site of application. Thus methylcholanthrene has been shown by many investigators to produce sarcomas as well as carcinomas at sites where it is applied. A few carcinogens produce tumors at a distance from the site of application and are usually site specific; e. g., β-napthylamine produces bladder tumors in dogs after ingestion (*4*). Ingested *p*-dimethylaminoazobenzene produces only liver tumors in rats, whereas a closely related compound, *o*-aminoazotoluene, when injected subcutaneously in oil solution into mice, elicits tumors at the site of application, tumors in the liver, lungs, and hemangio-endotheliomas at remote sites (*5*, *6*).

AAF was first reported by Wilson, De Eds, and Cox (*7*) to be a potent carcinogen when fed to the rat. Their observations have been amply substantiated by others (*3*, *8–16*) who have shown that AAF produces a variety of tumors located at sites distant from the portal of entry.

The present paper gives an account of the absorption and distribution of N-acetyl-2-aminofluorene (*17*).

The results indicated that, as suggested by the wide distribution of the tumors it induces, AAF was widely distributed in the body of the rat. All the carcinogen found in the blood occurred in the plasma where the highest concentration was observed a few hours, usually 2 to 4, after ingestion. High doses of the compound resulted in higher concentrations than lower doses. Highest concentrations were also found in liver and kidney at approximately the same interval as found in the plasma. Rapid inactivation of AAF in the rat was evidenced from the fact that organ and tissue concentrations were greatly decreased or in some cases no longer detectable well before the sixteenth hour, when considerable amounts still remained in the stomach contents.

MATERIALS AND METHODS

AAF, melting point 194° C., uncorrected, prepared by Dr. J. M. Johnson of the National Cancer Institute, was dissolved in warm coconut oil in

[1] Received for publication April 12, 1948.

[2] Presented in part before the Section of Biological Chemistry, American Chemical Society, September 1946, Chicago.

[3] Referred to as AAF.

[4] Bielschowsky (*3*) has reported that N-acetyl-2-amino-7 hydroxy fluorene isolated from the urine of rats ingesting N-acetyl-2-aminofluorene gives a slight color response following hydrolysis and diazotation when R salt (*1*) is used as the coupling agent.

a concentration of 6.25 mg. per milliliter. This solution was given by stomach tube at one of two dosage levels: A, 10.4 mg., and B, 3.1 mg. per 100 gm. body weight.

Several strains of albino rats were used in this study. Because it had been observed that coconut oil containing AAF in the amounts used had a purgative action in the fasted rat, the rats used in the experiments here reported were given free access to stock-diet food up to the time of feeding the coconut oil bearing AAF. It is unlikely that the quantity of food residue in the stomach was the same for the different tests. The male and female animals varied in both age and size because animals of uniform age, sex, and size were unavailable. While only rats of the same sex were used in any given test, there was more than a 100-percent variation in the weight of both males and females used in these experiments.

All rats were killed by asphyxiation. Before the heart stopped beating, the pericardial cavity was opened, the heart was exposed, and a slit was made with scissors. The blood was collected from the slit in oxalate and centrifuged to separate red cells from plasma. The other tissues and organs to be analyzed were removed and macerated in a Waring Blendor. Following maceration, the carcinogen was extracted with acetone and determined photometrically (*2*).

The procedure used to minimize contamination during removal of the organs and tissues for estimation of carcinogen was as follows: Removal of blood, brain, and leg muscle, followed by opening of the body cavity and removal of dorsal abdominal fat, the kidneys, and liver. After clamping off both openings to the stomach with hemostats, the intestines and, finally, the stomach were removed. The surgical instruments were immersed in acetone before use and after removal of each organ or tissue. Acetone is an excellent solvent for AAF.

Care was exercised at all times to avoid contamination of the tissues or organs with carcinogen. The greatest potential source for contamination of the various samples was from the contents of the stomach and intestines.

Analyses were made on the pooled samples from five rats for each organ or tissue regularly at 1-, 2-, 3-, 4-, 6-, 8-, and 16-hour intervals. In a few instances, shorter intervals were used near the beginning of the experiment.

EXPERIMENTAL RESULTS

Blood

All the AAF found in the blood was in the serum fraction, and no detectable amounts were found in the red cells at either level of feeding. A rapid rise in plasma AAF concentration was observed at the 10.4 mg./100 gm. body weight level of administration (fig. 1). This rapid rise continued for the first 3 hours at which time the the peak concentration of 450 μg. percent was attained. This was followed by a rapid fall in concentration for the next 2 hours and a gradual tapering off to the sixteenth hour when the concentration was 100 μg. percent. The plasma concentration in animals fed at 3.1 mg./100 gm. reached a very low peak after 3 hours, only one-tenth that found in the animals given the higher dosage. After the eighth hour no carcinogen was found (fig. 1, B).

Liver

Of the many tissues in which AAF induces tumors, the liver is the most susceptible as judged by the frequency of occurrence of tumors. It was found that at the 10 mg./100 gm. level of administration the highest concentration in the liver

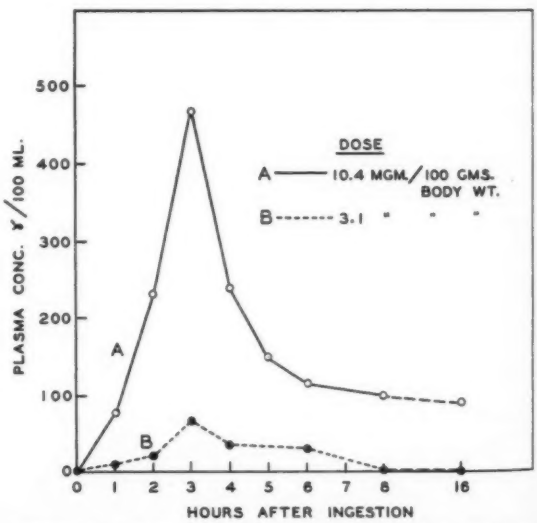

FIGURE 1.—Plasma concentration of ingested AAF at varying intervals.

occurred 2–3 hours after feeding, with the peak concentration reached at a maximum of about 900 μg. percent of fresh liver (fig. 2, A). The concentration decreased rapidly after 3 hours but somewhat less rapidly than it had increased during the first 3 hours. At 8 and 16 hours, less than 100 μg. percent AAF was found in the liver, a concentration somewhat less than that found in the plasma at corresponding time intervals.

There was no definite peak concentration found in the liver after feeding the lower dose (fig. 2, B). The highest concentrations were observed 1 to 3 hours after feeding. Only trace amounts were found in the liver of animals ingesting dose B at intervals of 6, 8, and 16 hours (fig. 2, B).

Kidney

The kidney is perhaps the main pathway utilized by the organism for excreting AAF. As in the plasma and liver, the peak kidney concentration occurred 3 hours after the administration of 10 mg./100 gm. and attained a concentration of 650 μg. percent, not quite so high as that found in the liver but higher than that found in the plasma.

As in the liver, no definite sharp peak of AAF concentration was found in the kidney at the lower dose (fig. 3, B). The concentration reached 150 μg. percent after the first hour and remained high until after the sixth hour. At the eighth and sixteenth hours, very small amounts were still found in the kidney indicating that the compound was still being excreted.

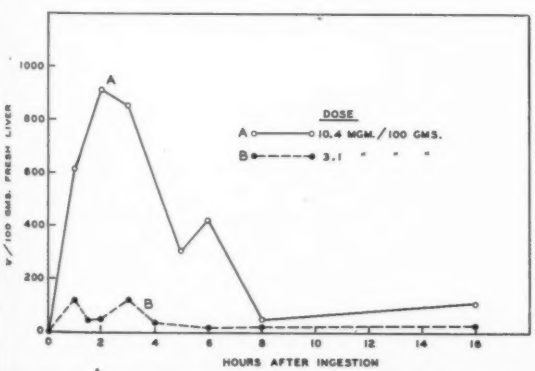

FIGURE 2.—The average concentration of AAF in fresh liver at varying intervals following ingestion.

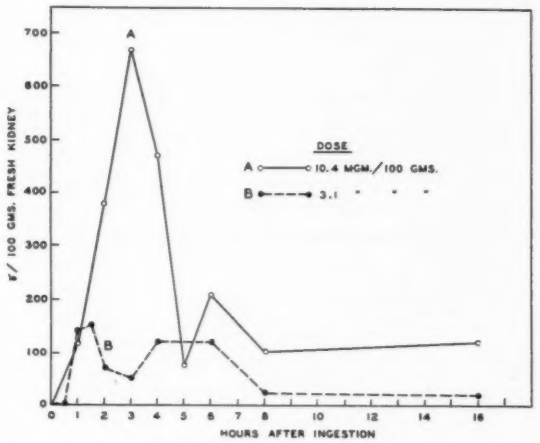

FIGURE 3.—The average concentration of carcinogen in the kidney of rats at varying intervals following ingestion.

Other Tissues

In this first survey of the distribution of N-acetyl-2-aminofluorene, it seemed important to determine how regularly the carcinogen could be found in some of the other tissues and at what concentrations. Three tissues, brain, dorsal abdominal fat, and muscle were selected. Muscle is relatively low in lipids, while the other two tissues are relatively high. AAF, like many other carcinogens, is much more soluble in fat or fat solvents than in water.

The concentration of carcinogen in the brain 1 hour after ingestion of dose A was 250 μg. percent and reached a maximum of over 300 μg. percent at 2 hours (fig. 4). The concentration remained high through 6 hours, then dropped rapidly to zero by the eighth hour. At the lower dose of 3.1 mg./100 gm., however, only a trace remained after the sixth hour (fig. 4).

A much slower rise in AAF concentration in the dorsal abdominal fat was found after 10.4 mg./100 gm., reaching a maximum of 100 μg. percent after 5 hours (fig. 4). This level was still present at the sixteenth hour. No carcinogen was found in the dorsal abdominal fat at any time, however, when the animal ingested 3.1 mg. AAF/100 gm. body weight (fig. 4).

The concentration of AAF in the leg muscle after the higher dose was lower than in any of the other tissues or organs examined, reaching a peak of about 50 μg. percent at 3 hours and then

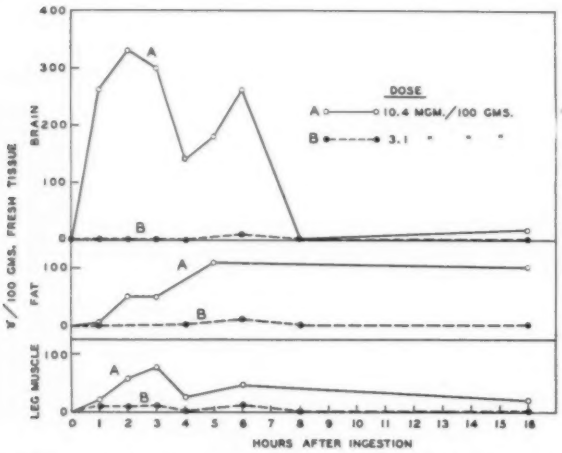

FIGURE 4.—Average concentration of AAF in brain, leg muscle, and dorsal abdominal fat at varying intervals following ingestion.

gradually tapering off so that after 16 hours only a trace remained (fig. 4). At the lower dose only low concentrations were obtained in the muscle, and these remained for a 6-hour period.

STOMACH

One of the most interesting and perhaps significant observations brought out in this experiment was that even after 16 hours 7 to 25 percent of the ingested dose was recovered from the stomach (table 1). An average of 38 percent of the ingested dose was present in the stomach 1 hour after administration of the smaller dose, while the recovery from the stomach after the larger dose was 54 percent at 1 hour, 71 percent at 4, and 67 percent at 5 hours (cf. table 1 and fig. 5).

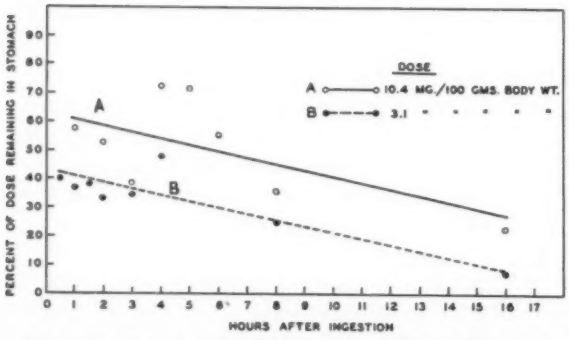

FIGURE 5.—Percent of the amount of AAF ingested which remained in the stomach after varying intervals.

The recovery of AAF from the stomach was considerably higher 4 and 5 hours after ingestion than it was 1 to 3 hours after ingestion of 10.4 mg./100 gm. The explanation for the exceptionally high values for the 4- and 5-hour periods is not apparent from these experiments, but some of the possible factors involved are the total quantity of fat administered, the amount of food residue in the stomach at the time of AAF ingestion, the size of the rat, and the known influence of fat on the emptying time of the stomach.

The recovery of oil from the stomach of the rats after ingestion of coconut oil containing the AAF fluctuated widely. Thus, 1 hour after the ingestion of the AAF-oil preparation at 3.1 mg./100 gm., the percentage of the ingested oil recovered from the stomach in two experiments was 40 and 75 percent (table 1).

TABLE 1.—*Influence of time on recovery of oil and AAF from the stomach of rats*

DOSE A (10.4 MG. PER 100 GM. BODY WEIGHT)

Experimental period (in hours)	Average body weight	Average dose per rat	Total oil intake per group	Total AAF intake per group	Total oil recovered from stomach		Total AAF recovered from stomach	
	Gm.	*Ml.*	*Ml.*	*Mg.*	*Ml.*	*Percent*	*Mg.*	*Percent*
1	216	3.6	10.8	67.5	7.3	68	36.5	54
2	228	3.8	11.4	71.5	8.3	73	33.2	47
2	288	4.9	24.0	150.0	16.3	69	91.4	61
2	304	5.0	25.1	157.0	12.4	49	74.5	47
3	212	2.8	14.1	88.0	8.9	63	33.8	38
4	187	3.1	15.6	98.0	10.7	69	66.7	67
5	176	3.0	15.0	94.7	11.6	77	67.0	71
6	147	2.5	12.4	77.5	7.1	57	42.8	55
8	260	4.2	21.2	132.5	11.6	55	46.4	35
16	355	6.0	29.8	185.0	10.3	35	45.5	25
DOSE B (3.1 MG. PER 100 GM. BODY WEIGHT)								
0.5	175	0.9	4.35	27.0	2.8	64	9.8	36
1.0	209	1.1	5.3	33.0	2.1	40	11.0	33
1.0	256	1.3	6.4	40.9	4.8	75	17.3	43
1.5	146	.8	3.8	23.7	3.0	79	9.0	38
2.0	190	.9	4.7	29.0	3.2	68	9.6	33
3.0	224	1.2	5.8	38.0	2.5	43	------	34
4.0	226	1.1	5.5	34.4	2.8	51	17.0	49
8.0	227	1.1	5.7	35.5	2.9	51	8.7	25
16.0	221	1.1	5.5	34.4	.8	15	2.4	7

The data reveal a slow emptying time of the stomach for both the oil and the carcinogen, and in general, as would be expected, there was a decrease in the amount of carcinogen remaining in the stomach from the beginning to the end of the 16-hour period (fig. 5).

DISCUSSION

It should be noted that in rats fed small doses of AAF (3.1 mg./100 gm.) no detectable amounts

of AAF were found in the plasma, muscle, dorsal abdominal fat, or brain after the eighth hour, even though there was a large reservoir of carcinogen still remaining in the stomachs of the animals after that interval. Small but detectable amounts of AAF, however, were present in the liver and also in the kidney of these dose B animals. This suggests that the animal is able to metabolize or in some way dispose of an appreciable amount of the carcinogen. The persistence of AAF in the liver and kidney after it had disappeared from the blood plasma is noteworthy, however.

The AAF as determined in these experiments was only that portion extractable by acetone. If part of the carcinogen enters into chemical combination with some body constituent, there is a possibility that it would not be removed by the extraction procedure used. Although the carcinogen was actually determined as the 2-aminofluorene, it does not appear in the animal as such, because hydrolysis by a strong acid (*2*) is required prior to its estimation.

The distribution of AAF throughout the tissues of the rat in comparatively high concentrations seems to be a logical explanation for the production of many tumors in this species at sites distant from the portal of entry.

It should be noted that the AAF was administered in oil solution. The influence of the quantity of fat administered to fasted rats (when expressed in percent of the administered fat which disappeared from the gastrointestinal tract) was studied by Deuel et al. (*18*), who found that the absorption was inversely proportional to the quantity administered. They also found a greater absorption of fat by large fasted rats than by small ones. Further, the well-known inhibitory effect of fat (*19*, *p. 484*) on the motor activity of the stomach would seem to be an important factor affecting the amount of recovery of both oil and AAF from this organ.

The observation that at low doses of carcinogen considerable quantities can be present in the stomach with none detectable in some of the tissues may explain why fewer tumors arise in rats ingesting low quantities of the carcinogen (*3*, *16*).

SUMMARY

A study of the concentration of N-acetyl-2-aminofluorene in organs of the rat over a 16-hour period after oral administration indicates a wide distribution of substantial amounts of this compound throughout the body.

After a dose of 3.1 mg. per 100 gm., 7 percent remained in the stomach after 16 hours while 25 percent remained after a dose of 10.4 mg. per 100 gm.

Detectable quantities of the carcinogen were found in identical tissues after both high and low (10.4 mg. and 3.1 mg. per 100 gm. body weight) oral doses of N-acetyl-2-aminofluorene. The highest concentration in plasma, liver, kidney, brain, and muscle was reached 3 hours after administration; in dorsal abdominal fat at the higher dosage, however, the peak concentration was not reached till 5 hours after ingestion.

At dosages of 3.1 mg./100 gm., the carcinogen persisted in the tissues for periods varying from 6 hours in the muscle to 16 hours in the liver and kidney. At the higher dosage of 10.4 mg./100 gm. demonstrable levels persisted for 8 to 16 hours.

REFERENCES

(*1*) Westfall, B. B.: Estimation of 2-aminofluorene and related compounds in biologic material. J. Nat. Cancer Inst. 6: 23–29 (1945).

(*2*) Westfall, B. B., and Morris, H. P.: Photometric estimation of N-acetyl-2-aminofluorene. J. Nat. Cancer Inst. 8: 17–22 (1947).

(*3*) Bielschowsky, F.: The carcinogenic action of 2-acetyl-aminofluorene and related compounds. Brit. M. Bull. 4: 382–385 (1947).

(*4*) Hueper, W. C., Wiley, F. H., and Wolfe, H. D.: Experimental production of bladder tumors in dogs by administration of beta-naphthylamine. J. Indust. Hyg. & Toxicol. 20: 46–84 (1938).

(*5*) Andervont, H. B., Grady, H. G., and Edwards, J. E.: Induction of hepatic lesions, hepatomas, pulmonary tumors, and hemangio-endotheliomas in mice by *o*-aminoazotoluene. J. Nat. Cancer Inst. 3: 131–153 (1942).

(*6*) Law, L. W.: The cancer producing properties of azo compounds in mice. Cancer Research 1: 394–401 (1941).

(*7*) Wilson, R. H., De Eds, F., and Cox, A. J.: The toxicity and carcinogenic activity of 2-acetaminofluorene. Cancer Research 1: 595–608 (1941).

(*8*) Bielschowsky, F.: Distant tumours produced by 2-amino and 2-acetyl-amino-fluorene. Brit. J. Exper. Path. 25: 1–4 (1944).

(*9*) BIELSCHOWSKY, F.: Comparison of the tumours produced by 2-acetyl-amino-fluorene in piebald and Wistar rats. Brit. J. Exper. Path. 27: 135–139 (1946).

(*10*) ———: The effect of a diet containing yeast upon the development of tumours induced by 2-acetyl-amino-fluorene. Brit. J. Cancer 1: 146–151 (1947).

(*11*) KIRBY, A. H. M.: The combined action of 2-acetyl-aminofluorene and sex hormones in the Wistar rat. Brit. J. Cancer 1: 68–79 (1947).

(*12*) DUNNING, W. F., CURTIS, M. R., and MADSEN, M. E.: Induction of neoplasms in five strains of rats with acetylaminofluorene. Cancer Research 7: 134–140 (1947).

(*13*) HEIMAN, J., and MEISEL, D.: Tumors of the salivary and parathyroid glands in rats fed with 2-acetyl-aminofluorene. Cancer Research 6: 617–619 (1946).

(*14*) CANTAROW, A., PASCHKIS, K. E., STASNEY, J., and ROTHENBERG, M. S.: Influence of sex hormones upon the hepatic lesions produced by 2-acetamino-fluorene. Cancer Research 6: 610–616 (1946).

(*15*) MORRIS, H. P., DUBNIK, C. S., DUNN, T. B., and JOHNSON, J. M.: Tumors produced in rats after ingestion or painting of 2 nitro, 2 amino, N-acetyl-2-amino, and N-diacetyl-2-aminofluorene. Cancer Research 7: 730–731 (1947).

(*16*) WILSON, R. H., DE EDS, F., and COX, J. A.: Carcinogenic activity of 2-acetaminofluorene. II. Effects of concentration and duration of exposure. Cancer Research 7: 444–449 (1947).

(*17*) MORRIS, H. P., DUBNIK, C. S., DUNN, T. B., and WESTFALL, B. B.: Some observations on carcinogenicity, distribution, and metabolism of N-acetyl-2-aminofluorene in the rat. 4th Internat. Cancer Research Congress, St. Louis, September 2–7, 1947.

(*18*) DEUEL, H. J., Jr., HALLMAN, L., and LEONARD, A.: Comparative rate of absorption of some neutral fats. J. Nutrition 20: 215–226 (1940).

(*19*) BEST, C. H., and TAYLOR, N. B.: Physiological basis of medical practice. Williams & Wilkins, Baltimore (1945).

CARBONIC ANHYDRASE CONTENT OF NORMAL AND NEOPLASTIC TISSUES[1]

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INTRODUCTION

Carbonic anhydrase, which catalyzes both the formation of carbonic acid from carbon dioxide and water and the reverse reaction, is found in high concentration in red blood cells (*1*) and is present in many other tissues, notably gastric mucosa and renal cortex (*2*). It is absent from normal plasma, urine, skin, and lungs (*2*). The enzyme is a zinc-containing protein (*3*) and apparently takes part in the acid-base metabolism of the body (*4*).

Hodgson (*5*) found no marked changes in the carbonic anhydrase content of the blood of two patients with cancer. Ashby (*6*) reported a reduced concentration of carbonic anhydrase in the muscle of cancerous as compared with noncancerous individuals. Leiner (*7*) compared the carbonic anhydrase content of several tissues of normal rats with the corresponding tissues in rats bearing transplanted Jensen sarcoma and Flexner-Jobling carcinoma. No differences were observed, although the tumors themselves had lower enzyme activity than the normal tissues studied.

There is apparently an acid shift in the hydrogen-ion concentration in hepatic tumors (*8*). It was postulated that carbonic anhydrase may be involved in this shift. An investigation of the carbonic anhydrase content of normal and neoplastic tissues in rats and mice, as well as in rat livers during the precancerous stage, was therefore undertaken.

EXPERIMENTAL PROCEDURE

Sixty young, adult, male rats of the Wistar strain, weighing between 150–200 gm., were placed on the corn-oil diet of Kline et al. (*9*), with the omission of the carcinogen and with the substitution of sucrose for cerelose. After a 1-month equilibration period, 0.06 percent *p*-dimethylaminoazobenzene was added to the diet of 40 of the animals for 120 days, while the remaining 20 animals continued on the control diet. Two experimental animals were killed every 2 weeks, and 2 control animals were killed every 4 weeks for enzyme assays. None of the experimental animals developed hepatomas in 6 months, although atypical cells compatible with early neoplastic changes were observed in histologic section of 2 livers.

Five hepatoma-bearing Sprague-Dawley male rats were therefore obtained.[3] The animals had been fed a semisynthetic diet containing 0.06 percent *p*-dimethylaminoazobenzene for 4½ to 5 months. The livers of all bore one or more tumors. The hepatic tumors were of the mixed type described by Edwards and White (*10*); in one rat the tumor was disseminated widely throughout the peritoneal cavity. Two Sprague-Dawley females of the same stock, fed the same diet without carcinogen, served as controls.

Two strains of mice were used: Strain L mice bearing transplanted liver tumor C954, a vascular adenocarcinoma probably derived from biliary epithelium,[4] and strain A mice bearing either a transplanted mammary adenocarcinoma or a transplanted sarcoma.[5]

The animals were killed either by exsanguination or by cervical dislocation. Homogenates (*11*) were prepared in cold distilled water from the livers of the rats and from the liver, kidneys, glandular portion of the stomach, and tumors of the mice. In hepatoma-bearing rats homogenates were prepared from grossly nonnecrotic areas of the tumors and from adjacent uninvolved tissue. All homogenates were diluted to 25 cc. with distilled water and were used for the estimation of carbonic anhydrase, nitrogen (*12*), and residual blood (*13*). Carbonic anhydrase was determined essentially by Keilin and Mann's modification of the method of Philpot and Philpot (*14*).

One milliliter of 0.026M sodium bicarbonate was added to a 25 x 200-mm. reaction tube, followed by the desired amount of enzyme preparation, 0.2 ml. of 0.04 percent phenol red, and one

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drop of caprylic alcohol. The mixture was then diluted to 11 ml. with cold distilled water. For control runs, the enzyme preparation was omitted. The reaction tube was immersed in a water bath maintained at a temperature of 0°–1° C. Carbon dioxide was passed through the reaction mixture at a constant rate of flow, while the mixture was stirred mechanically. After the carbon dioxide had passed through the solution for 2 minutes, 2 ml. of a cold standard carbonate-bicarbonate solution, containing 40 ml. of 0.52M sodium bicarbonate and 50 ml. of 0.60M sodium carbonate per 100 ml. was added, and the time required for acidification of the solution was determined. An enzyme unit is defined as that amount of carbonic anhydrase which decreases the acidification time to one-half that required for control determinations.

Blood was collected for the determination of carbonic anhydrase and hemoglobin (*15*). In calculating the carbonic anhydrase content of the tissues, corrections were always applied for the enzyme content of the residual blood. In some instances, corrections were also made for the nitrogen contributed by the residual blood.

TABLE 1.—*Carbonic anhydrase in blood and livers of normal rats*

Rat No.[1]	Time of death [2]	Weight of liver	Ratio of liver weight to body weight	Hemoglobin in blood (grams per 100 cc.)	Carbonic anhydrase in—		
					Blood		Liver
					Units per cubic centimeter	Units per milligram hemoglobin	Units per milligram nitrogen [3]
	Weeks	*Grams*	*Percent*				
1C	0	8.8	4.1	15.7	560	3.6	1.98
2C	0	11.0	4.6	15.4	340	2.2	1.62
3C	0	10.7	5.0	15.2	440	2.9	1.80
4C	0	9.1	4.0	14.8	570	3.8	1.88
5C	0	8.6	4.1	15.0	430	2.9	1.48
6C	4	7.9	2.9	14.7	290	2.0	1.12
7C	4	10.7	3.8	15.2	310	2.1	1.39
8C	8	11.6	4.1	15.0	440	3.0	1.02
9C	8	11.5	3.6	14.3	340	2.3	1.18
10C	12	11.8	3.9	13.5	380	2.9	1.45
11C	12	15.1	4.5	13.8	450	3.3	1.60
12C	16	14.3	4.5	13.6	430	3.2	1.80
13C	16	11.3	3.9	14.5	530	3.6	1.51
14C	20	16.8	5.7	13.2	450	3.4	1.44
15C	20	13.8	5.8	12.6	470	3.7	1.37
20C	.....	.....	.....	16.5	300	1.8	1.31
21C	.....	.....	.....	15.6	370	2.4	1.57

[1] Rats 1C through 15C, Wistar strain; rats 20C and 21C, Sprague-Dawley strain.
[2] Following end of equilibration period.
[3] Uncorrected for nitrogen in residual blood. Corrected values, determined for rats 20C and 21C, were approximately 5 percent higher.

TABLE 2.—*Effect of p-dimethylaminoazobenzene on carbonic anhydrase in rats*

Rat No.[1]	Time of death [2]	Weight of liver	Ratio of liver weight to body weight	Hemoglobin in blood (grams per 100 cc.)	Carbonic anhydrase in—			
					Blood		Liver	Hepatoma
					Units per cubic centimeter	Units per milligram hemoglobin	Units per milligram nitrogen [3]	Units per milligram nitrogen [3]
	Weeks	*Grams*	*Percent*					
1E	2	9.8	4.2	12.2	340	2.8	1.42	.....
2E	2	9.8	4.4	13.0	310	2.4	1.77	.....
3E	4	8.0	4.1	13.5	340	2.5	1.72	.....
4E	4	9.2	4.2	12.1	360	2.9	1.19	.....
5E	6	5.8	2.9	13.0	230	1.7	1.21	.....
6E	6	11.7	6.5	12.5	320	2.5	1.27	.....
7E	8	10.0	4.4	13.1	300	2.3	1.54	.....
8E	8	9.6	4.7	12.4	280	2.2	1.32	.....
9E	10	8.4	4.4	12.3	530	4.3	1.11	.....
10E	10	7.5	4.1	12.5	300	2.4	1.23	.....
11E	12	8.4	5.2	11.6	230	2.0	1.30	.....
12E	12	7.0	3.6	13.1	310	2.4	1.36	.....
13E	14	6.1	3.9	14.3	380	2.7	1.54	.....
14E	14	6.1	3.4	12.6	600	4.7	1.80	.....
15E	16	7.2	3.4	13.0	420	3.3	1.63	.....
16E	16	9.5	4.6	13.0	450	3.5	1.50	.....
17E	18	9.2	4.1	14.1	420	3.0	1.16	.....
18E	18	10.2	4.4	13.2	340	2.6	1.31	.....
19E	20	10.0	4.3	16.9	790	4.7	1.48	.....
20E	20	9.5	3.5	16.2	590	3.7	1.39	.....
21E	22	7.4	3.4	15.3	400	2.6	1.38	.....
22E	22	14.6	7.3	12.6	360	2.8	1.42	.....
23E	26	7.6	3.3	13.8	360	2.6	1.03	.....
24E	26	17.7	6.0	12.6	280	2.2	1.24	.....
25E	26	14.0	7.0	16.9	430	2.5	1.52	.....
29E	26	13.8	6.9	13.8	320	2.3	1.12	.....
30E	26	15.0	6.4	13.8	420	2.3	1.51	.....
34E	26	14.3	6.1	12.4	360	2.9	1.35	.....
40E	.....	39.2	21.8	13.9	380	2.7	2.41	[4] 1.33 [4] 1.48
41E	.....	9.3	6.5	13.8	300	2.2	2.20	.45
42E	.....	49.6	26.5	.....	.....	3.2	1.69	1.10
43E	.....	43.5	16.1	13.5	210	1.5	1.13	[5] .58 [6] .51
44E	.....	17.2	9.3	16.2	300	1.8	2.29	.50

[1] Rats 1E through 34E, Wistar strain; Rats 40E through 44E, Sprague-Dawley strain.
[2] Following end of equilibration period.
[3] Uncorrected for nitrogen in residual blood; corrected values determined for rats 40 through 44E were approximately 5 percent higher.
[4] From tumors of approximately equal size.
[5] Grom a large tumor.
[6] From a small tumor.

RESULTS

Rats

The carbonic anhydrase content of the tissues examined is shown in tables 1 and 2. The enzyme concentration in blood, both on a volume and on a hemoglobin basis, varied considerably. In the control animals, the carbonic anhydrase content of whole blood roughly paralleled that of liver. This correlation was less marked in the experimental animals.

No changes were observed in the liver carbonic anhydrase of animals fed *p*-dimethylaminoazobenzene, as compared with control animals, before hepatoma development. When the criterion of Opie (*16*) of ratio of liver weight to total body

weight was used, several of the experimental animals had developed hepatic hyperplasia. Moreover, on histologic examination large atypical cells were found in the livers of animals 25E and 29E, indicating incipient hepatoma formation; yet the enzyme concentrations were unchanged. It would appear, therefore, that administration of the azo dye does not lead to changes in liver carbonic anhydrase content during the preneoplastic stage.

In animals bearing hepatomas, the carbonic anhydrase content of the tumor and of the residual liver appeared to depend on the extent of the neoplastic involvement. Thus in animals 41E and 44E, both of which had relatively small livers with few tumors, the uninvolved areas of liver were higher and the tumor areas lower in enzyme concentration than were livers of control animals. In animals 42E and 43E, where the livers were greatly enlarged, with multiple large and small tumors, the nonneoplastic areas of liver had a normal enzyme content, while the hepatoma areas of rat 43E were low in enzyme content. In rat No. 40E, which also had a markedly enlarged liver with many tumor areas, the carbonic anhydrase value of the uninvolved portions of the liver was elevated, while the tumor areas showed a normal enzyme level.

The tumors of all animals had lower carbonic anhydrase concentrations than the adjacent nonneoplastic areas. In a given animal, the size of the tumor apparently did not affect its enzyme concentration. No marked differences in enzyme content were found in large as compared with small tumors.

Mice

The carbonic anhydrase content of tissues of normal mice and of mice bearing transplanted tumors is shown in table 3. It is apparent that the corresponding tissues of the normal and of the tumor-bearing animals, with the exception of liver, were comparable in carbonic anhydrase concentration, as Leiner (7) had previously found with rats. The enzyme content of the livers of tumor-bearing animals appeared to be lower than normal. In mice as with rats the carbonic anhydrase concentration of liver roughly paralleled that of blood. Since the tumor-bearing animals were markedly anemic, reduced levels of enzyme in both blood and liver were not surprising. No significance is therefore attached to the slightly lower levels of carbonic anhydrase in the livers of tumor-bearing animals. The findings suggest the possibility of a dynamic equilibrium between blood and liver carbonic anhydrase, the liver, perhaps, serving as a source of the enzyme in blood.

The three tumors investigated showed considerable variation in carbonic anhydrase content. Mammary carcinoma and sarcoma tissues had lower enzyme contents than the normal tissues assayed, while the liver tumor showed an enzyme

TABLE 3.—*Carbonic anhydrase in tissues of normal and of tumor-bearing mice*

Strain	Group	Hemoglobin in blood (grams per 100 cc.)	Carbonic anhydrase in—						
			Blood			Liver	Kidney	Stomach	Tumor
			Units per cubic centimeter	Units per milliliter hemoglobin	Units per milligram nitrogen	Units per milligram nitrogen [1]	Units per milligram nitrogen [1]	Units per milligram nitrogen [2]	Units per milligram nitrogen [1]
L	Control	17.1	280	1.6		1.26		8.5	
		18.2	420	2.3	11.6	1.57	3.2	8.2	
		17.5	360	2.1	9.6	1.05	3.1	6.9	
		17.6	410	2.3	11.8	1.38			
	Liver tumor	5.1	90	1.8		.46			1.22
		4.8	110	2.3	5.8	1.04	3.2	6.9	1.27
		2.5	82	3.3	7.4	.67	4.1	8.0	.97
		11.7	280	2.4	10.3	1.14	3.7	9.2	.92
		16.4	410	2.5	12.2	1.64	5.4	10.2	
A	Control	14.9	260	1.7	7.6	1.19	3.1	4.0	
		15.2	270	1.8	7.6	1.08			
	Mammary carcinoma	12.8	440	3.4	9.7	.69	3.5	4.6	.13
		12.5	310	2.4	11.8	1.18			.44
	Sarcoma	8.6	200	2.3	8.3	.75	3.7	5.2	.60
		10.4	280	2.6	9.8	.93			.70

[1] Uncorrected for nitrogen in residual blood; corrected values where determined were approximately 5 percent higher.
[2] No measurable quantities of residual blood were found in the stomach.

concentration comparable with that of the liver of the host. It is apparent, therefore, that tumors like normal tissues vary considerably in carbonic anhydrase content.

SUMMARY

No significant changes were observed in the carbonic anhydrase content of the livers of rats fed *p*-dimethylaminoazobenzene prior to the malignant transformation. Primary liver tumor induced by administration of the azo dye contained approximately 50 percent of the carbonic anhydrase present in normal rat liver.

The carbonic anhydrase concentration of three types of transplanted mouse tumors varied with the type of tumor. The growth of the tumors had no apparent effect on the enzyme content of several tissues of the host.

It is suggested that the liver may serve as the source of blood carbonic anhydrase.

REFERENCES

(*1*) Meldrum, N. U., and Roughton, F. J. W.: Carbonic anhydrase, its preparation and properties. J. Physiol. 80: 113–142 (1933).

(*2*) Davenport, H. W.: Carbonic anhydrase in tissues other than blood. Physiol. Rev. 26: 560–573 (1946).

(*3*) Keilin, D., and Mann, T.: Carbonic anhydrase. Purification and nature of the enzyme. Biochem. J. 34: 1163–1176 (1940).

(*4*) Feldberg, W., Keilin, D., and Mann, T.: Activity of carbonic anhydrase in relation to gastric secretion. Nature, London 146: 651–652 (1940).

(*5*) Hodgson, T. H.: The carbonic anhydrase content of blood in pathological states in man. Brit. J. Exper. Path: 17: 75–80 (1936).

(*6*) Ashby, W.: Carbonic anhydrase in mammalian tissue. J. Biol. Chem. 151: 521–527 (1943).

(*7*) Leiner, M.: Der Kohlensäureanhydratasegehalt in Geweben und im Blut. Naturwissenschaften 31: 468–469 (1941).

(*8*) Kahler, H., and Robertson, W. v. B.: Hydrogen-ion concentration of normal liver and hepatic tumors. J. Nat. Cancer Inst. 3: 495–501 (1943).

(*9*) Kline, B. E., Miller, J. A., Rusch, H. P., and Baumann, C. A.: The carcinogenicity of *p*-dimethylaminoazobenzene in diets containing the fatty acids of hydrogenated coconut oil or of corn oil. Cancer Research 6: 1–4 (1946).

(*10*) Edwards, J. E., and White, J.: Pathologic changes with special reference to pigmentation and classification of hepatic tumors in rats fed *p*-dimethylaminoazobenzene (butter yellow). J. Nat. Cancer Inst. 2: 157–183 (1941).

(*11*) Potter, V. R., and Elvehjem, C. A.: A modified method for the study of tissue oxidations. J. Biol. Chem. 114: 495–504 (1936).

(*12*) Koch, F. C., and McMeekin, T. L.: A new direct nesslerization micro-Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. J. Am. Chem. Soc. 46: 2066–2069 (1924).

(*13*) Klein, J. R.: Estimation of blood in tissue. Arch. Biochem. 8: 421–424 (1945).

(*14*) Philpot, F. J., and Philpot, J. St. L.: A modified colorimetric estimation of carbonic anhydrase. Biochem. J. 30: 2191–2193 (1936).

(*15*) Evelyn, K. A., and Malloy, H. T.: Microdetermination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. J. Biol. Chem. 126: 655–662 (1938).

(*16*) Opie, E. L.: Mobilization of basophile substance (ribonucleic acid) in the cytoplasm of liver cells with the production of tumors by butter yellow. J. Exper. Med. 84: 91–105 (1946).

AN INFILTRATING ADENOMATOUS LESION OF THE COLON OF RATS INGESTING MOTOR LUBRICATING OIL (S. G. F. NO. 1 OIL)[1,2]

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INTRODUCTION

Following the unexpected and rapid development of hyperplastic polypoid gastritis in monkeys exposed to an aerosol of S. G. F. No. 1 oil (*1*), rats were similarly exposed in order to determine whether the labile gastric mucosa of this species (*2*) would be susceptible to injury by the oil. The striking similarity of the gastric lesion of the monkey to that produced in rats fed various fats and oil by Roffo (*3*, *4*) encouraged us to study also the effects of feeding the oil to rats.

In the course of the experiments reported here, it was learned that the S. G. F. No. 1 oil,[4] is not a crude shale oil as previously reported (*1*). This oil is actually a highly refined diesel engine lubricating oil obtained from gulf coast naphthenic crude base oil and known commercially as "Diol",[5] Likewise in error in that paper was the observation that this oil contained "substances with the absorptive spectra of carcinogenic hydrocarbons." By using a new, well calibrated spectrophotometer and the more modern techniques of Berenblum (*5*), this oil was shown to have an ultraviolet absorption spectrum indicative apparently of a lack of any substances similar to the known carcinogens.[6] Figure 1 is a chart comparing the absorptive curve of this oil with that of 3, 4-benzpyrene, a typical naturally occuring carcinogen.

EXPERIMENTAL PROCEDURES

Two experiments were performed. In the first, 240 albino Wistar rats[7] were used. They averaged 250 gm. in weight and were about 6 months of age. They were divided into 6 groups of 40 rats each. Groups 1, 2, and 3 were fed a diet similar to that fed by Roffo (*3*), consisting of finely ground dried white bread and liquid whole milk. Groups 4, 5, and 6 were fed finely ground laboratory dog chow and water. The diet of half of the animals in each group was supplemented twice each week with fresh cabbage. The diet of groups 2 and 5 contained 0.015 and 0.0113 ml. of oil per gram of ground diet, respectively, so that on the basis of daily food consumption each rat ingested on the average 0.20 ml. of oil per day. Groups 3 and 6 were exposed 12 hours each day to a fog of the oil consisting of 60 micrograms of oil per liter of air, generated as described in the previous experiment with monkeys (*1*). For the first 6 months, 2 rats in each group were killed bimonthly in hopes of observing the sequence of events in the formation of any gastrointestinal lesions. When dead, moribund, or at the end of 14 months, the remaining animals were necropsied, and sections were made of the stomach, small intestine, colon, liver, kidney, spleen, pancreas, mediastinal and abdominal lymph nodes, lungs, and bone marrow.

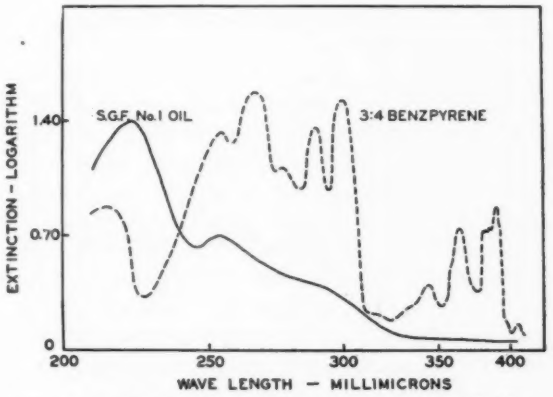

FIGURE 1.—Ultraviolet absorption curves of S. G. F. No. 1 oil and 3,4-benzpyrene, a typical naturally occurring carcinogen, compared to show in the former the apparent lack of substances with absorptive spectra like the carcinogenic hydrocarbons.

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[2] Part of this work was done under contract between the University of Chicago Toxicity Laboratory and the Medical Division, Chemical Corps, U. S. Army, which neither restricts nor is responsible for the opinions or conclusions of the authors.

[3] This study depended in a large measure upon the diligent care of the animals by Hannah Berry, the accuracy of the hematologic determinations of Benjamin Jacques, and the innumerable excellent histologic preparations of Charles Blinn. The authors gratefully acknowledge the long-continued kindly advice and encouragement of Dr. Paul R. Cannon and Dr. Eleanor Humphreys and their indebtedness to Dr. Harold L. Stewart for studying this material with them.

[4] United States Army Chemical Corps designation of oil used for smoke screens.

[5] Manufactured by Standard Oil Co. of New Jersey and by other companies under various trade names.

[6] This study was made by Dr. Hans L. Falk, Department of Pathology.

[7] Obtained from Maguran Farms, Michigan.

The second experiment was done to determine whether the incidence of the lesions found in the colons of the rats in the first experiment (table 1) could be increased. Since many of the animals in the first experiment died from pneumonia and leukemia, Sprague-Dawley rats were used in the second experiment because of their apparently greater resistance to these diseases. The coexistence of macrocytic hyperchromic anemia, atrophic gastritis, and gastric carcinoma in human beings has pointed toward the presence of common etiological factors in these diseases (*2*, *6*, *7*, *8*). Assuming that the nutritional deficiency in pernicious anemia predisposes the gastric mucosa to injury and neoplastic change, an attempt was made in the second experiment to determine whether the susceptibility of the stomach of the rat to injury might be increased by causing a folic acid deficiency concomitantly with the ingestion of the oil. This deficiency was produced by method of Black et al. (*9*), using a synthetic diet [8] containing 1 percent succinylsulfathiazole (SST). The 158 rats used in this experiment were 6 weeks old and weighed 100 gm. They were divided into 4 groups: Group 1, 36 rats, ingested this diet without the SST; group 2, 35 rats, ate the same diet as group 1 to which was added 0.015 ml. of oil per gram of diet; group 3, 53 rats, fed on the diet containing the SST; and group 4, 34 rats, ate the same diet as group 3 but also containing the same amount of oil as the diet of group 2. All diets were fed ad libitum, but the average daily consumption per rat was as follows: Group 1, 16 gm.; group 2, 8 gm.; group 3, 13 gm.; and group 4, 7 gm. During the first 3 months of this experiment determinations were made at weekly intervals of the total numbers of erythrocytes and leukocytes and the hemoglobin and hematocrit percentages of the peripheral blood of 8 rats selected at random from each group. These determinations were repeated thereafter at about biweekly intervals for 3 additional months. It was felt unwise after that time to subject the few remaining rats in groups 2 and 4 to the trauma of bleeding since they were very ill and emaciated. Because 8 animals ingesting the SST and oil died during the first 3 months of this experiment and the remainder on this diet appeared extremely ill, an attempt was made to prolong the lives of half of these rats by placing them on the control diet for 8 weeks, after which they were returned to the diet containing SST and oil. After 50 days on the diets, 5 rats in each group were killed for histologic studies as in the first experiment. Thereafter only dead or moribund animals were necropsied until the end of 270 days, when, after all animals in groups 2 and 4 had died, the 5 remaining rats in group 3 were killed along with 5 rats from group 1. The remaining 16 rats in group 1 were kept under observation on the control diet in order to determine the incidence of neoplasia and other disease in this strain of rats.[9]

[8] Composition of diet: Casein, 18 percent; dextrin, 57 percent; SST, 1 percent; Ruffex, 5 percent; Mendel's salt mixture, 4 percent; cod-liver oil, 2 percent; Mazola oil, 2 percent; and water, 10 percent. Vitamins in milligrams per gram of diet were: thiamine, 0.1; pyridoxine, 0.1; riboflavin, 0.2; calcium pantothenate, 0.2; niacin, 1.0; choline chloride, 20.0; and to half of the animals in each group, biotin, 0.5.

RESULTS

In experiment 1, the oil failed to produce any gastric changes. All deviations from the normal could be attributed to the effects of malnutrition; as for example, occasional instances of hyperplastic epithelial change and superficial ulcerations in the forestomachs. Most of the animals that died succumbed to pneumonia and pulmonary abscesses. Cannibalism kept about 50 percent of the animals from being necropsied. No changes were seen in the intestinal tract until the final killing after 14 months of feeding the diet (table 1). Among the animals killed then, a microscopic focus of hyperplastic and slightly atypical glands was

TABLE 1.—*Effect of S. G. F. No. 1 oil on rats fed the diet of Roffo (3)*

Group [1]	Rats alive at months—			Rats necropsied after 6 months		Rats with leukemia	Rats with lesion in colon [3]
	6	12	14 [2]	At death	Total		
1	34	17	16	10	26	3	0
2	33	7	3	15	18	3	1
3	33	19	15	12	27	3	2
4	35	18	15	13	28	0	0
5	34	8	1	18	19	3	1
6	34	18	16	11	27	2	1

[1] 40 rats in each group: group 1, diet of white bread and whole milk; group 2, same diet plus oil; group 3, same as group 1, but exposed to oil fog; group 4, dog chow and water; group 5, same diet plus oil; group 6, same diet as group 4, but exposed to oil fog.
[2] Time of final killing and examination.
[3] Lesions in colon: group 2, focus of hyperplastic colonic mucosal glands; group 3, 1 adenocarcinoma with metastases to regional lymph node and 1 reticular-cell sarcoma; group 5, ulcerative cecitis; group 6, focus of hyperplastic colonic mucosal glands.

[9] These rats were 15 months old on May 1, 1948. No neoplasms or clonic lesions had developed up to that time.

found in the mucosa of the first 4 cm. of the colon in one rat from the group eating the bread and oil and another in a rat eating the chow in the oil exposure chamber. One animal on the bread diet in the oil exposure chamber had a primary reticular-cell sarcoma of this portion of the colon where another in the same group had a primary polypoid adenocarcinoma with metastases to a regional lymph node. The latter neoplasm was similar to those in this location in human beings. The polypoid portion above the muscularis mucosae consisted chiefly of abnormal hyperplastic, cystic, and tortuous glands. As the epithelial cells infiltrated through the submucosa, they became poorly differentiated and pleomorphic and formed imperfect glands. They penetrated the muscle coats of the intestine as cords of undifferentiated cells. In a regional lymph node, several foci of signet-ring carcinoma cells were found. One rat eating the chow and oil had typical ulcerative cecitis as described by Stewart and Jones (*10*).

Table 2 summarizes the results of the second experiment and tabulates the occurrence of the various lesions. Feeding the oil in the synthetic diet without the SST resulted in the occurrence of an unusual lesion in the colon of 50 percent of the animals necropsied later than 120 days after the beginning of the diet. This lesion, which at first glance appeared neoplastic both on gross and histologic examination, was not found in the animals of any other group. As far as is known it has never been described before. Six animals in group 4 ingesting the oil and the SST did not develop this lesion although they ate as much oil as the animals with the lesion in group 2. Figure 2 shows the amount of oil ingested up to the time of death by individual rats in these two groups, comparing the incidence and time of occurrence of the colonic lesions. About an equal number of hyperplastic intramucosal foci were found in the

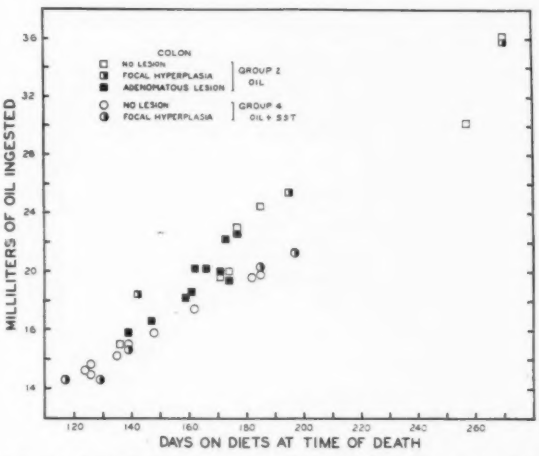

FIGURE 2.—Oil ingested up to time of death by rats fed diet with oil (group 2), and diet with oil and SST (group 4), comparing incidence and time of occurrence of colonic lesions.

TABLE 2.—*Effect of ingesting S. G. F. No. 1 oil and/or succinylsulfathiazole on the incidence and time of appearance of colonic lesions in rats fed synthetic diets*

Numerator, number of focal hyperplastic lesions
Denominator, number of adenomatous lesions
Integral number, rats dying during 10-day interval

Group [1]	Number of rats	Rats alive at 120 days	Deaths and incidence of colonic lesions by days (grouped by 10-day intervals)															Total number of lesions and deaths between 120 and 270 days
			120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	
1	36	31	.....	.....	.....	.....	.....	.....	$\frac{0}{0}$ 5 [2]	.....	$\frac{0}{0}$ 1	.....	.....	.....	.....	.....	$\frac{0}{0}$ 1	$\frac{0}{0}$ 5 [2]	$\frac{0}{0}$ 28 [3]
2	35	30	.....	.....	$\frac{0}{1}$ 2	$\frac{1}{1}$ 2	$\frac{0}{1}$ 1	$\frac{0}{3}$ 3	$\frac{0}{4}$ 7	$\frac{0}{0}$ 1	$\frac{1}{0}$ 1	.....	.....	.....	.....	.....	$\frac{0}{0}$ 1	$\frac{1}{0}$ 2	$\frac{3}{1}$0 20
3	55	46	.....	$\frac{0}{0}$ 2	$\frac{1}{0}$ 5	$\frac{0}{0}$ 1	$\frac{0}{0}$ 4	$\frac{0}{0}$ 5	0	$\frac{0}{0}$ 3	$\frac{0}{0}$ 3	$\frac{0}{0}$ 2	$\frac{0}{0}$ 1	$\frac{0}{0}$ 1	$\frac{0}{0}$ 7	.....	.....	$\frac{0}{0}$ 5 [2]	$\frac{1}{0}$ 39
4	34	22	$\frac{1}{0}$ 1	$\frac{1}{0}$ 4	$\frac{1}{0}$ 3	$\frac{0}{0}$ 1	.....	$\frac{0}{0}$ 1	.....	$\frac{1}{0}$ 3 [4]	$\frac{1}{0}$ 1 [4]	.....	.....	.....	.....	.....	.....	.....	$\frac{5}{0}$ 14

[1] Group 1, control diet; group 2, diet with oil; group 3, diet with SST; group 4, diet with oil and SST. Loss of rats due to cannibalism: group 1, 3; group 2, 10; group 3, 7; and group 4, 8.
[2] Animals killed.
[3] 16 animals surviving, being observed for incidence of natural disease.
[4] Animals in group 4 fed control diet between the eighty-fourth and one hundred and fortieth day in order to prolong life.

colons of both groups. No changes other than the usual effects of malnutrition were found in the stomachs of these animals. No atrophic gastritis was found in the animals ingesting the SST with or without the oil.

All the intestinal lesions were distributed within the first 4 cm. of the colon distal to the cecum as in the first experiment. The commonest location was midway in this segment at about the site of the first focus of submucosal lymphatic tissue of the colon. The neoplastic-like lesion appeared as a sharply demarcated pinkish-white nodular swelling to which omentum, mesentery, and pancreas often adhered. Occasionally this tumor was tubular, appearing as a uniformly thickened segment of bowel 1–2 cm. in length. Figure 3 shows a section of a tubular lesion. The nodular lesions felt solid, but on opening the colon the lumen in the involved segment was found irregularly enlarged by sacculations. The walls of these sacculations were stiffened and greatly thickened by firm pink tissue. The mucosal surfaces were covered by relatively large papillary projections or by ragged necrotic tissue.

Histologically, the lesions were similar to those in the stomachs of the monkeys exposed to an aerosol of the oil (*1*) except that in most of the rats the proliferating mucosal glands penetrated through all of the muscular layers and formed acini in the peritoneum and adhesions. The similarity was most apparent in the mucosal changes of the tubular lesions of the rats. Figure 4 is a higher power photomicrograph of an area in figure 3, illustrating the adenomatous type of hyperplasia, polyp formation, loss of the muscularis mucosae, and glandular growth down to but not into the circular muscle of the colon. These were typical features of the hyperplastic gastritis in the monkeys. The submucosal edema and fibrosis which were also prominent in the lesions of the monkeys were seen in only 2 of the 10 colonic lesions of the rat. The relatively little mucosal change accompanying this phenomenon may indicate that this is an early stage in the pathogenesis of the fully developed disease. Figure 5 shows the extreme submucosal edema and the penetration of the still intact muscularis mucosae by cyst-forming glands from the apparently normal mucosa. The preservation of continuity with the superficial mucosa is well illustrated. In more advanced cases where the glands had penetrated into the serosal inflammatory tissue, this continuity was still maintained as shown by semi-serial sections of the lesions.

Large areas of coagulation necrosis were present in many of the lesions. Figure 6 shows such an area in the midst of an adenomatous colon. The epithelium marked by the arrow is normal. Just to the right of this is a relatively large diverticulum. Diverticula of this nature occured in conjunction with the necrosis and inflammation that was present in most of the lesions. Typically, these diverticula had walls made up of mesentery or omentum and granulation tissue covered by purulent exudate and necrotic debris. No remnants of muscle were found in their walls. In the lesion in figure 6, besides a typical polyp, there are also a pancreatic adhesion to the large necrotic area and two foci of gland formation in the chronic granulation tissue of the serosa. The necrosis in these lesions was apparently secondary to the inflammation and infection in the hyperplastic mucosa. In some superficial necrotic areas the coagulated remnant of the adenomatous mucosa was observed. This necrosis occurred only after the circular muscle was infiltrated by the glands and a serosal inflammation had developed. In figures 7, 8, and 9 the stages in this process are illustrated. In the low-power photomicrograph (fig. 7), early necrosis of the colon is seen on the

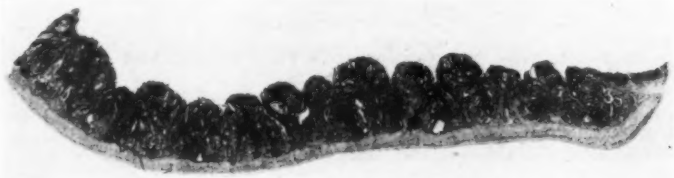

FIGURE 3.—Section of the tubular lesion of the colon of rat F–41, dead after 138 days of ingesting oil, showing the uniform thickening of the wall by adenomatous hyperplasia of the muscoa. × 10.

FIGURE 4.—Area from figure 3, a lesion of the tubular variety, showing the adenomatous hyperplasia, pseudo-polyp formation, loss of muscularis mucosae, and proximity of the proliferating glands to the circular smooth muscle. × 60.

left. This is the oldest area of the lesion as evidenced by the advanced penetration of the walls of the intestine by the glands, seen here chiefly as cystic spaces at the level where the muscle coats have been completely destroyed. There are two diverticula in this area, the smaller of which has remnants of a glandular epithelial lining. The portion of this section to the right is entirely involved but the disease is not as advanced as in the area of necrosis. In figures 8 and 9, taken from the marked areas in figure 7, the earlier stages can be seen. In figure 8, there is a focus of hyperchromatic glands penetrating the circular muscle. These do not contain much if any exudate while the superficial glands do. To the right of this focus the muscle has been penetrated by four glands which are filled with cellular exudate and debris. Most of the intraluminal exudate seems to stem from the cystic glands of the superficial portions. In figure 9, other examples of this process are to be seen along with break-through of the lining epithelium of the intramuscular glands and resulting abscess formation, destruction of muscle and myositis. The adenomatous hyperplasia in these illustrations is typical of that in most of the lesions. Occasional foci of this hyperplastic process assumed a more malignant appearance of neoplastic nature as in figure 10.

Examination of the regional lymph nodes in all cases failed to reveal any metastases. The lesion of rat F–71, dead after 163 days on the oil-containing diet, was interpreted as further evidence of the nonneoplastic nature of this lesion. While this lesion had large typical areas of necrosis, the remaining mucosa differed markedly from that of the lesions of the other animals. In figure 5 an apparently early stage in the development of this lesion in this rat is depicted. Adjacent to the necrotic area there were many large polypoid projections, but much of the mucosa around and over

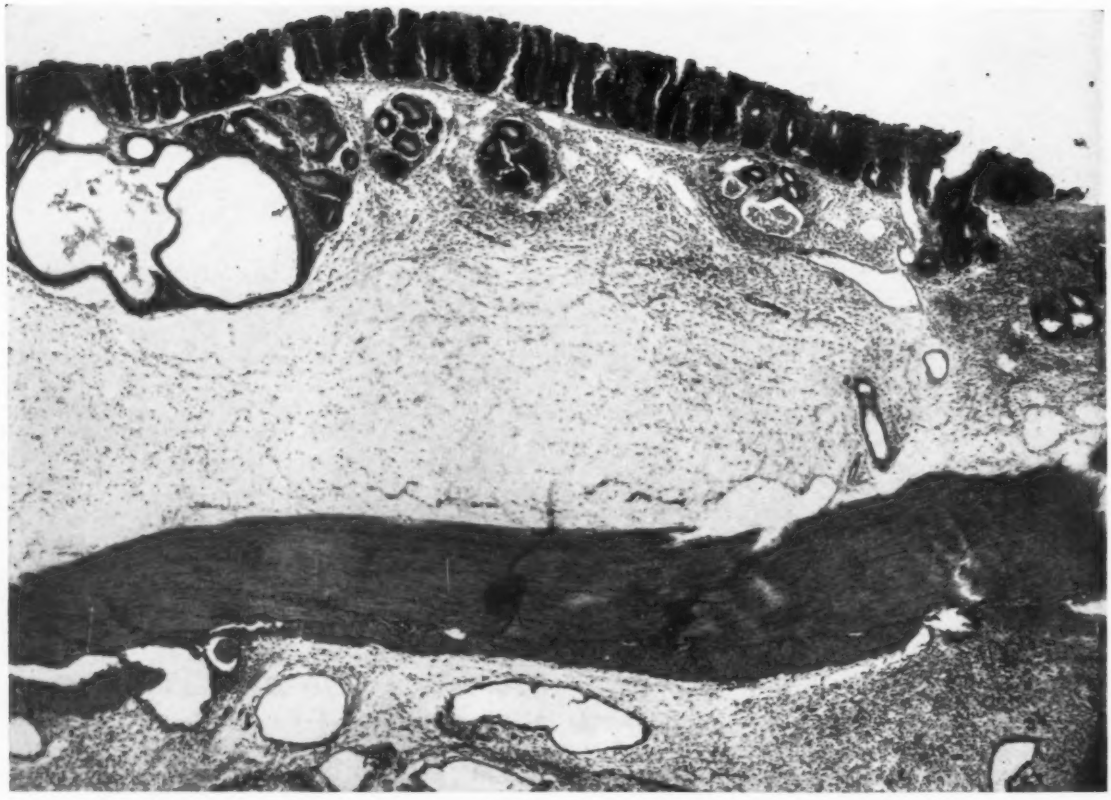

FIGURE 5.—Early lesion from rat F–71, dead after 163 days, showing marked submucosal edema and fibrosis, the penetration of the muscularis mucosae by cystic glands, and the relatively normal mucosa. × 60.

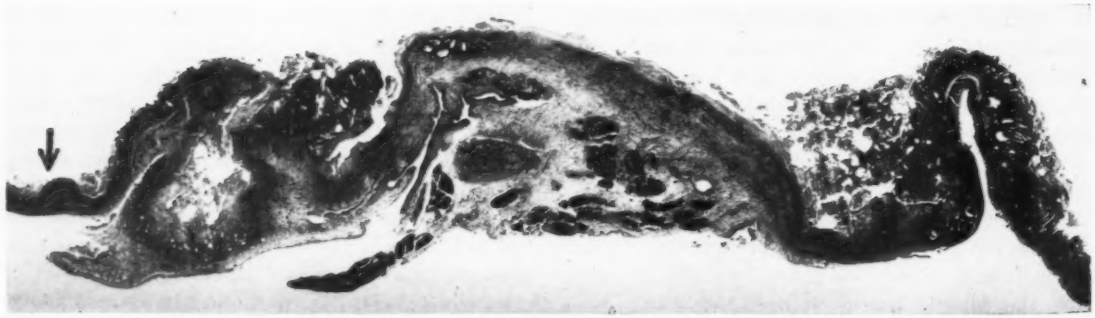

FIGURE 6.—Colonic lesion of rat F–58, dead after 146 days, showing (arrow) normal epithelium, a diverticulum, a large pseudo-polyp, a necrotic ulcer, and a pancreatic adhesion. × 10.

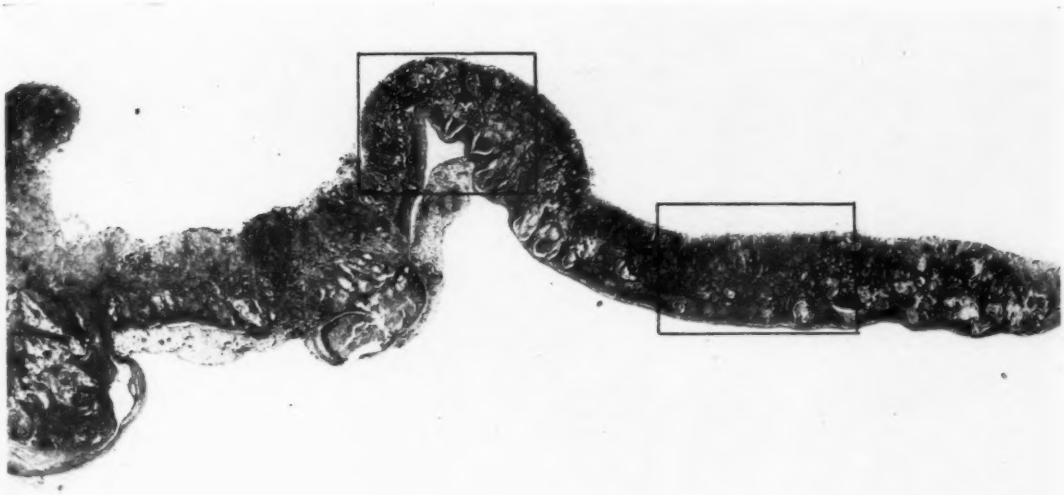

FIGURE 7.—Lesion of rat F–79, dead after 173 days on oil diet, showing early necrosis, two diverticula, and adenomatous hyperplasia, with numerous instances of penetration of the muscle by glands. × 10.

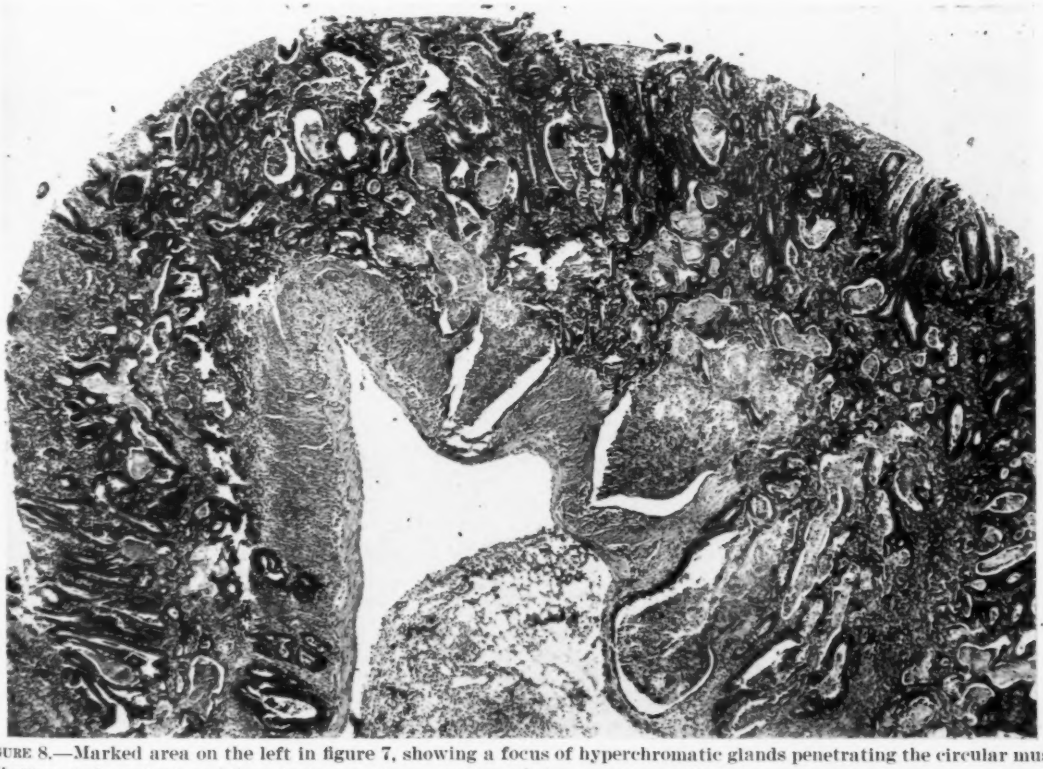

FIGURE 8.—Marked area on the left in figure 7, showing a focus of hyperchromatic glands penetrating the circular muscle (bottom, left center) and four instances of penetration of the muscle and dilatation of the glands by cellular exudate and debris. × 60.

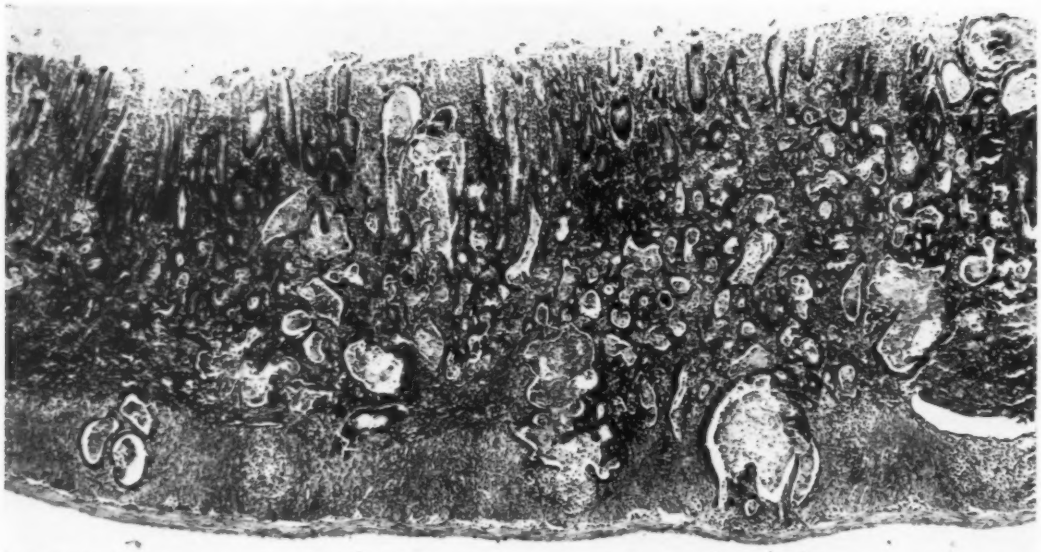

FIGURE 9.—Marked area on the right in figure 7, showing the apparent extension of the intraluminal inflammatory process through the epithelium of the penetrating glands into the adjacent smooth muscle.

FIGURE 10.—Lesion in rat F–84, dead after ingesting oil 176 days, showing the very disorderly arrangement of the infiltrating hyperplastic glands that give the appearance of neoplasia. × 60.

these "polyps" was as thin as normal. The character of this portion of the colon is illustrated in figure 11. Figure 12 is a higher power photomicrograph of the labeled polypoid area in the preceding figure. It shows that the apparently normal thin mucosa is actually atrophic and that the size and form of the polyps depend in a large measure on clumps of hypertrophic smooth muscle in their centers. The first polyp on the left, while adenomatous, does not appear to have as active or disorderly a mucosa as that in other lesions. The most logical interpretation of this change seems to be that the active processes of hyperplasia and downward spread of the glands and inflammation with destruction of the muscle coats has subsided in this area and has been succeeded by atrophy of the mucosa and hypertrophy of the muscular remnants.

The focal muscosal hyperplasia seen in this experiment in the colons of 3 animals ingesting oil, 5 animals ingesting oil and SST, and 1 animal ingesting SST without oil, was similar to that seen in the first experiment. This lesion consisted of a focus of from 5 to 20 mucosal glands that were larger, darker staining, and more crowded with cells than the normal mucosal glands. Such a focus can be seen in figure 13, a photomicrograph of a section of the unopened colon of rat F–56 dead after ingesting oil and SST for 163 days. Although logically this focal hyperplasia might appear to be the earliest form of the large infiltrating adenomatous lesions, too few instances of different degrees of this change were found to permit reconstruction of the course of events.

Examination of the other tissues of these animals revealed, in addition to the acute and chronic

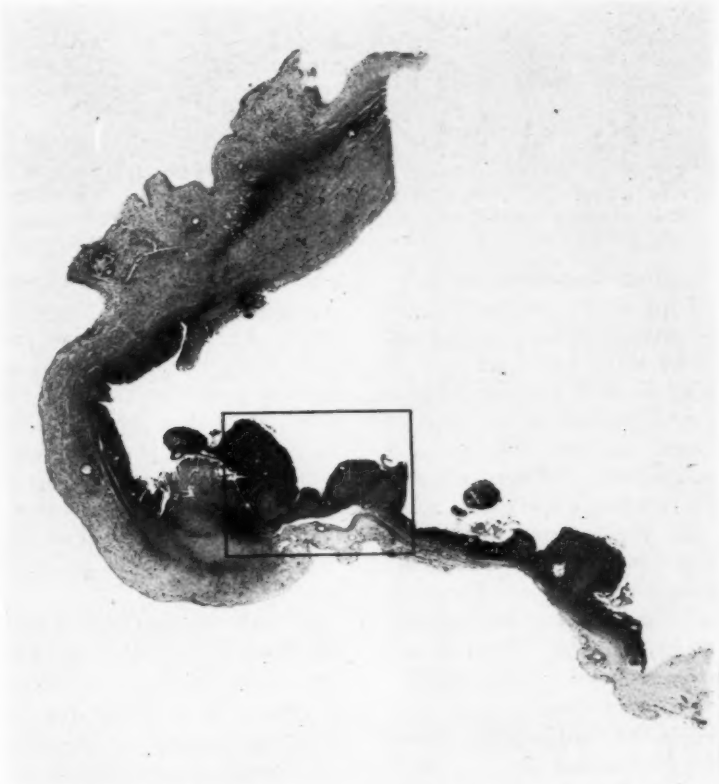

FIGURE 11.—Lesion of rat F–71, dead after 163 days on the oil diet, showing areas of thin mucosa and peculiar polypoid projections adjacent to several foci of necrosis. × 10.

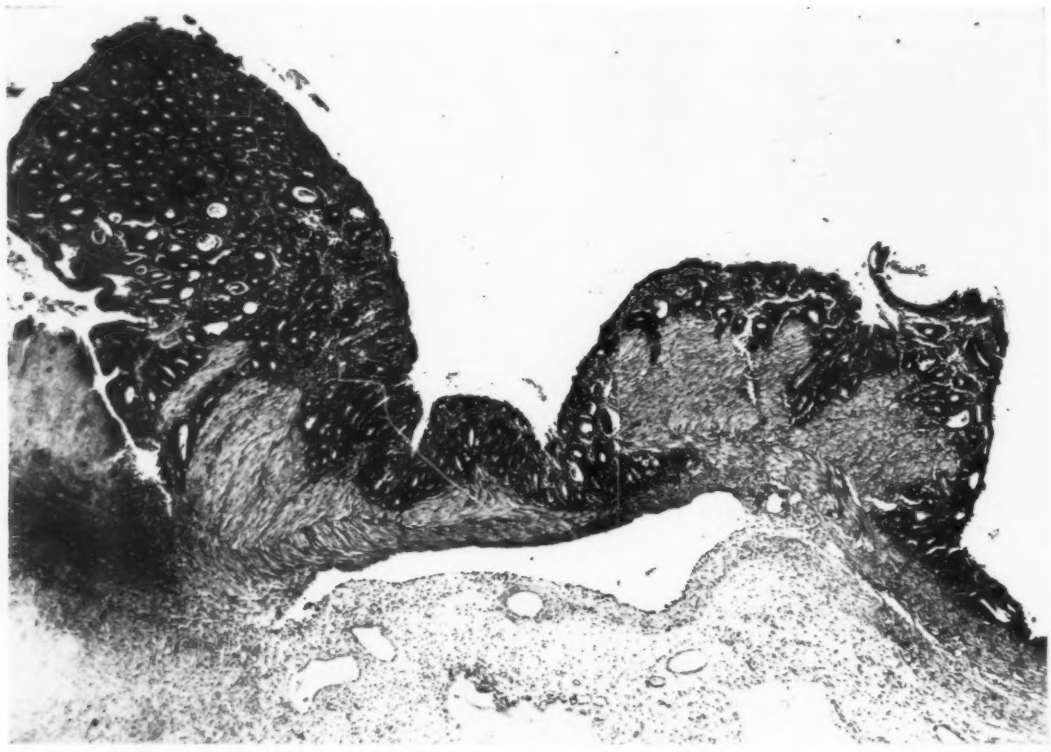

FIGURE 12.—Designated area in figure 11, showing the mucosal atrophy and hypertrophy of remnants of smooth muscle that may have resulted following cessation of the active process. × 60.

pneumonias that were often the cause of death, focal accumulations of the oil in the livers of the group with the adenomatous colonic lesions and usually a severe atrophy of the bone marrow in all animals except those of the control group. In the group ingesting the SST without the oil, most of the rats dying between 130 and 190 days had atrophy of the bone marrow. Between 190 and 230 days most of the rats had hyperplastic marrows, while after that the marrow was again atrophic. The changes in the red pulp of the spleens paralleled those in the marrow. The other changes reported in animals fed sulfaguanidine (*11*, *12*, *13*), such as hyperplastic thyroidal and arterial changes, were not found. Occasional instances of hepatic hydropic degeneration (*13*) were observed. Atrophy of the lymphatic tissue of the animals on the experimental diets was often extreme as would be expected following such severe malnutrition. The extent of the severe malnutrition can be seen in figure 14, which shows the average gain in weight of the animals on the various diets. The combination of the oil and SST was more toxic than either substance alone and caused the lowest consumption of the diet. Oil was more debilitating than the SST alone, which produced surprisingly little restriction in growth. The toxicity of the diets was reflected well in the death rates. Before 110 days, at which time table 2 starts, eight rats had died on the oil and SST, three on SST, none on oil, and one on the control diet.

In the accompanying hematologic investigation, the effects of the SST without the oil were not very noticeable when the average response of the group was determined. Individual variation in susceptibility was extreme. A few rats gained weight very slowly and became anemic and leukopenic very early in the experiment. These rats were the first to die. Most of the animals on this diet main-

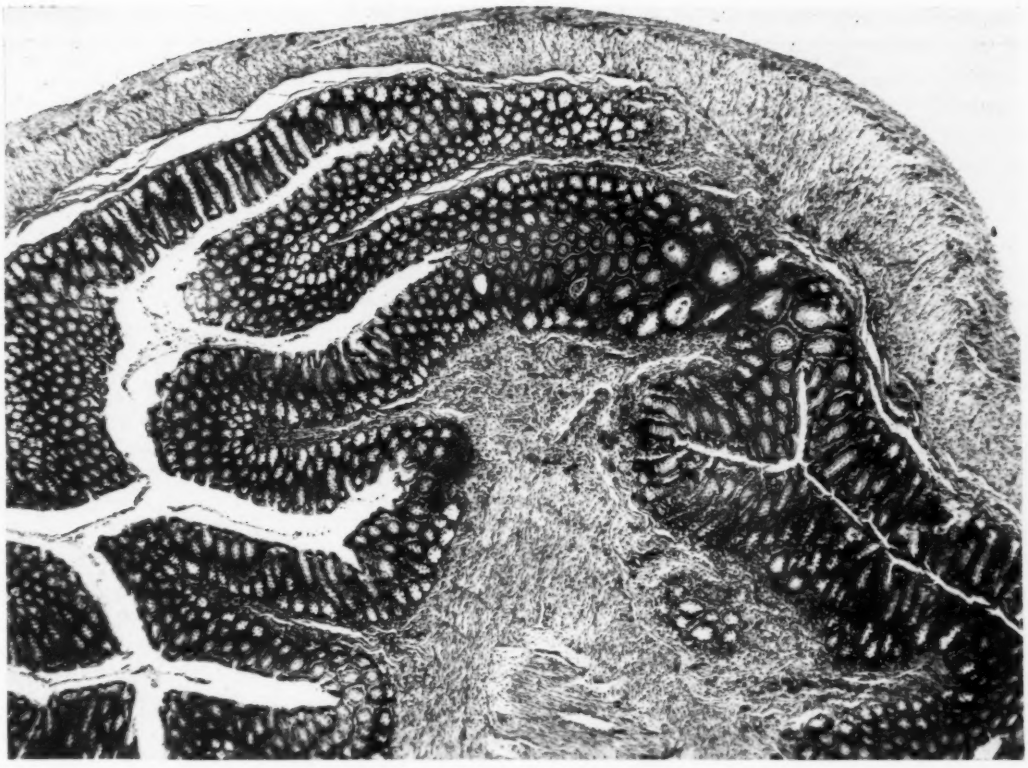

FIGURE 13.—Unopened colon of rat F–56, dead after 163 days of oil and SST, showing a focal area of hyperplastic hyperchromatic intramucosal glands. Compare the normal mucosa in this section with the adenomatous mucosa of rats fed oil alone, in figures at the same magnification. × 60.

tained a relatively normal peripheral blood picture until shortly before death. The oil caused a moderate reduction in numbers of circulating leukocytes after the first month. This effect was enhanced by the concomitant feeding of SST. Most of the changes in the blood picture of these animals and those on the oil can be explained on the basis of low protein intake (*14*). A macrocytic hyperchromic anemia was found in the group on oil during the thirteenth and fifteenth weeks and in the group on SST without oil during the thirteenth week on the diet. This was not seen in the other two groups. The group on SST and oil had a normo- or microcytic anemia with normo- or hypochromic cells throughout the course of the experiment. Table 3 is a summary of the mean hematologic values of the peripheral blood of the rats in this experiment. No correlation was found between the time of occurrence, duration and severity of the anemia or leukopenia with the development and extent of individual colonic lesions. The most

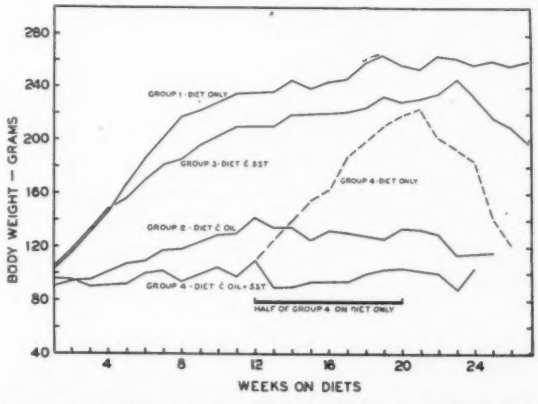

FIGURE 14.—Mean body weights of rats in each group during course of second experiment, showing growth restriction of diets of groups 2 and 4.

TABLE 2.—*Mean hematologic values*[1] *of the peripheral blood in rats fed lubricating oil and succinylsulfathiazole*

Weeks	WBC[2]				RBC[2]				Hb.[2]				Ht.[2]				M. C. V.[2]				M. C. H.[2]			
	Groups[3]				Groups[3]				Groups[3]				Groups[3]				Groups[3]				Groups[3]			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0	11.0	11.8	10.0	12.4	5.8	6.6	6.3	5.4	13.8	14.3	14.7	14.9	.....	.....	.....	.....	...	...	...	...	23.8	21.4	23.4	27.6
1	13.2	12.2	11.8	16.1	6.2	7.7	6.7	7.6	14.9	16.4	14.2	16.8	.....	.....	.....	.....	...	...	...	...	24.0	21.3	21.2	22.1
2	16.2	13.6	12.6	13.4	8.0	8.5	8.1	9.6	14.3	14.9	15.2	15.6	43.4	45.1	44.3	44.1	54	53	55	46	17.9	17.5	18.8	16.5
3	16.0	9.6	13.6	12.3	7.4	8.1	7.3	8.1	14.0	13.4	14.2	14.3	40.0	38.1	37.4	39.7	54	47	51	49	18.9	16.6	19.4	17.7
4	14.4	9.6	11.1	10.8	7.4	8.2	6.9	8.1	15.7	13.9	14.6	13.9	44.6	43.4	42.6	41.6	60	53	62	51	21.2	17.0	21.2	17.2
5	13.0	8.0	9.8	8.8	7.7	7.6	7.9	7.6	14.9	14.4	15.3	13.9	42.5	40.1	42.4	40.8	55	53	54	54	19.4	19.0	19.4	18.3
6	17.9	10.3	7.6	8.6	8.1	7.9	8.0	7.5	13.8	12.7	14.6	13.5	35.8	42.2	36.2	38.7	44	53	45	52	17.0	16.1	18.3	18.0
7	13.3	8.3	8.8	6.8	8.1	7.7	8.2	7.9	14.3	12.9	13.8	12.5	44.3	31.6	41.6	29.1	55	41	51	37	17.7	16.8	16.8	15.8
8	17.0	7.4	7.2	6.2	8.3	8.1	7.0	6.7	14.8	13.0	14.0	11.9	40.0	36.8	39.1	34.5	48	45	56	52	17.8	16.1	20.0	17.8
10	13.2	8.4	7.8	4.6	9.2	7.2	7.6	7.0	15.6	14.1	14.7	12.6	36.4	33.6	31.6	31.0	40	47	42	44	17.0	19.6	19.3	18.0
11	13.8	8.5	10.1	3.6	9.3	8.5	6.7	6.5	16.4	14.2	14.5	12.1	41.1	37.4	35.8	31.1	44	44	53	48	17.6	16.7	21.6	18.6
13	13.3	8.0	9.7	4.4	8.6	5.7	5.6	5.9	17.0	14.1	14.6	11.7	39.6	40.7	39.2	34.3	46	72	70	58	19.8	24.8	26.1	19.8
15	11.8	7.9	8.8	3.8	7.3	5.0	7.3	6.5	16.2	12.1	15.0	13.9	46.7	39.8	43.1	39.6	64	79	59	61	22.2	24.2	20.6	21.4
17	11.1	7.9	10.6	6.3	8.6	6.6	8.0	7.5	16.1	13.3	15.0	14.4	47.6	31.8	45.9	43.3	55	48	57	58	18.7	20.2	18.8	19.2
19	12.6	.....	7.9	6.8	7.4	....	6.9	7.3	16.2	.....	14.6	13.4	43.6	.....	40.6	33.2	59	...	59	46	22.0	.....	21.2	18.4
21	.....	5.3	8.0	.....	7.4	....	5.8	5.1	16.2	.....	12.9	12.5	43.4	28.7	39.5	.....	59	...	68	...	22.0	.....	22.2	24.4
22	.....	.....	6.2	.....	7.6	....	6.5	....	16.4	.....	13.6	.....	45.5	.....	37.5	35.2	60	...	58	...	22.0	.....	21.0	.....
24	12.3	2.6	8.8	.....	7.8	....	7.9	....	16.4	.....	15.1	.....	44.2	.....	42.1	.....	57	...	53	...	21.0	.....	19.1	.....

[1] Each figure is the average of the determination in 6–8 animals.
[2] WBC, leukocyte count in thousands per cubic millimeter; RBC, red cell count in millions per cubic millimeter; Hb., hemoglobin in grams per 100 ml. blood; Ht., volume of packed red cells in milliliters per 100 milliliters; M. C. V., mean corpuscular volume in cubic microns; M. C. H., mean corpuscular hemoglobin in micromicrograms.
[3] Group 1, control diet; group 2, diet with oil; group 3, diet with SST; group 4, diet with oil and SST.

severely anemic animals never had any gastric changes attributable to the SST or the oil. Thus, the answers obtained to the original questions that led to this investigation were: The gastric mucosa of the rat is not susceptible to injury by S. G. F. No. 1 oil and is not made more susceptible to injury or injured by a folic acid deficiency.

DISCUSSION

While we were attempting to learn whether or not S. G. F. No. 1 oil would injure the stomachs of rats, a hitherto undescribed epithelial lesion was produced in the colons of the animals fed the oil in addition to a basal synthetic diet. Colonic lesions have rarely been found in animals other than man. In a review of the literature concerning cancer of the alimentary canal of animals, Wells (*15*) reported 71 carcinomas of the intestinal tract of experimental and domestic animals and those dying in zoological gardens throughout the world. Of these carcinomas 2 were primary in the cecum and only 1 in the colon. Of the 70 carcinomas of the intestinal tract that have been reported since that review, only 2 were definitely adenocarcinomas of the colon (*16*, *17*) and not of the cecum, rectum, or small intestine. An additional instance of this rare lesion is the probably spontaneous adenocarcinoma of the colon of the rat in the first experiment reported in the present paper. The feeding of carcinogenic hydrocarbons often results in adenocarcinoma of the small intestine of mice (*18*, *19*) and rats (*17*, *20*). The large intestine is spared except in rats fed 2-amino or 2-acetylaminofluorene which, unlike most carcinogens, produces a great variety of tumors distant from the point of contact with the carcinogen. With this carcinogen all of 17 carcinomas of the large intestine (*17*) were primary in the cecum or rectum except 1 of the 2 mentioned above. In mice the carcinogen causing the neoplasms of the small intestine was found in the intestinal tract only as low as the ileocecal valve while a related but apparently noncarcinogenic substance was found in the colon (*18*). When mineral oil instead of a digestible oil was used as a vehicle for the carcinogen, these findings were not altered (*18*). Apparently the colons of animals are relatively well protected from carcinogenic and irritative influences in their lumina. The more frequent occurrence of carcinoma of the cecum and the common occurrence of ulcerative cecitis (*10*) in the rat indicate that the cecum is not so well-protected. In view of these facts, the high incidence of an infiltrating adenomatous lesion restricted to the colon just distal to the cecum of rats fed an oil apparently devoid of any known carcinogen is surprising.

The nature of this lesion is obscure. Whether or not it is a malignant neoplasm is not known. After these tumorlike masses were unexpectedly found at necropsy, repeated attempts were made unsuccessfully to palpate them in the remaining

living animals in order that transplantation of viable tissue could be tried. Since the natural course of the disease or its incidence was not known, random sacrifices for this purpose could not be justified in this experiment. In experiments now in progress, attempts to transplant this tissue are planned. The absence of any metastases and the relatively well differentiated epithelium of the most distant infiltrations seem to indicate that if this lesion is neoplastic, it is precancerous or a cancer of very low malignancy. The association of the adenomatous hyperplasia and suppurative inflammation, on the other hand, support the diagnosis of reactive hyperplasia. The apparently subsiding lesion (fig. 12) strengthens this view. The failure of this process to occur in the animals ingesting the succinylsulfathiazole and the oil may be the result of the bacteriostatic action of this drug. This would suggest that the lesion represents an epithelial reaction to chronic irritation enhanced by bacterial infection. The presence of epithelial glands infiltrating the submucosa and muscularis of the intestinal tract of animals has often been wrongly interpreted as indicative of malignant change. In a recent study of human regional enteritis,[10] examples were found where concomitant ulceration and epithelial hyperplasia led to apparent invasion of the walls of the intestine by glandular epithelium. In experiments now in progress, the deficiencies resulting from feeding SST and oil are being alleviated by the parenteral administration of folic acid to some animals and the additional feeding of *p*-aminobenzoic acid to others. It is hoped that this procedure will prolong the lives of the animals ingesting the oil and also enable evaluation of the roles infection and undernutrition undoubtedly play in the pathogenesis of this lesion. The minor role of the ingestion of the oil and the importance of other unknown factors in the production of this lesion are well-illustrated in the results that are being obtained in this current experiment. Although the conditions of the second experiment have been faithfully duplicated, no colonic adenomatous lesions have arisen in rats ingesting the oil alone, and in the other groups only one noninfiltrating adenomatous lesion has been found in the animals ingesting oil and *p*-aminobenzoic acid for 170 days.

[10] Humphreys, E.: Personal communication.

On the basis of the experiment reported here, the lesion apparently begins as scattered foci of intramucosal hyperplastic glands. Submucosal edema and inflammation occur allowing these glands to penetrate through faults in the stretched muscularis mucosae. The penetrating glands which maintain their continuity with the surface apparently allow bacterial invasion of the intestinal wall. The fibrous and muscular barriers to further extension of the hyperplastic epithelium are thereby weakened. Epithelial glands in the outer muscular coats become infected. These infected cystic glands break down, and muscle is destroyed in the inflammatory process. With this weakening of the wall diverticula develop but are prevented from rupturing by the thick serosal plaques and adhesions that form in such advanced areas. At about this stage, apparently because the infectious process has become fulminating, large areas of the adenomatous epithelium become necrotic and are sloughed. No vascular changes were found that could account for this necrosis. All of the animals died at or before this stage of the lesion. Evidence from the lesion of one animal seems to indicate that the process can subside spontaneously and leave an atrophic epithelium, pseudopolyps, and pseudodiverticula. From the similarity of the early stages of this lesion to those of the induced precancerous lesions of the small intestine of mice (*21*), it would seem also possible that this lesion ultimately might become malignant if infection and necrosis did not shorten the lives of the rats.

These experiments unfortunately do not contribute greatly to the understanding of the problem of carcinogenesis in the human colon. The human lesions most analogous to those described here are regional enteritis and the rare familial disease, colitis polyposa (*22, p. 509*). It is possible that human pseudodiverticulosis coli develops similarly to these diverticulous lesions. This adenomatous colonic lesion of the rat is morphologically almost identical to the gastric lesion reported in this species by Roffo (*3, 4*). They are so alike in fact that, lacking evidence that the colonic lesion is even precancerous, Roffo's claim that the gastric lesion is an adenocarcinoma seems unfounded, an opinion expressed by many others previously (*2, 23*).

SUMMARY

In two experiments rats were fed S. G. F. No. 1 oil to determine whether it would be injurious to the stomachs of this species. In the groups fed the oil in ground bread or dog chow, two rats developed foci of colonic mucosal hyperplasia and one colonic adenocarcinoma. In the second experiment, the oil was fed in a completely synthetic diet so that succinylsulfathiazole could be added and a folic acid deficiency produced. This was done to study the effect of this deficiency upon the susceptibility of gastric mucosa to injury. The oil did not cause any gastric changes in the rats with or without the succinylsulfathiazole but produced an adenomatous infiltrating mucosal lesion in the colons of half of the animals. The development of this lesion was apparently prevented by the concomitant feeding of the drug with the oil, but the occurrence of the focal intramucosal hyperplasia of the colons was not.

The adenomatous lesions which appear carcinomatous because of glands which infiltrate all layers of the intestinal wall are described and discussed. The tentative conclusion is that this lesion results from a hyperplasia of the mucosa reacting to chronic irritation and from an accompanying bacterial infection that destroys the fibrous and muscular barriers to heterotopic epithelial growth.

REFERENCES

(*1*) Lushbaugh, C. C.: Experimental hyperplastic gastritis and gastric polyposis in monkeys. J. Nat. Cancer Inst. 7: 313–320 (1947).

(*2*) Barrett, M. K.: Avenues of approach to the gastric cancer problem. J. Nat. Cancer Inst. 7: 127–157 (1947).

(*3*) Roffo, A. H.: Cancerización gastrica por el alquitran de cafe. Bol. Inst. de med. exper. para el estud. y trat. d. cáncer 20: 425–446 (1943).

(*4*) ———: Valor cancerigeno de los aceites oxidados. II. El aceite de girasol. Bol. Inst. de med. exper. para el estud. y trat. d. cáncer 21: 1–46 (1944).

(*5*) Berenblum, I., and Holiday, E. R.: Some physical methods of investigating carcinogenic hydrocarbons. Brit. M. Bull. 4: 326–330 (1947).

(*6*) Brown, M. R.: Pathology of the gastro-intestinal tract in pernicious anemia and subacute combined degeneration of spinal cord: study of one hundred and fifty-one autopsies. New England J. Med. 210: 473–477 (1934).

(*7*) Rhoads, C. P.: Gastric cancer as a sequel to gastritis, particularly the gastritis of pernicious anemia. J. Nat. Cancer Inst. 1: 511–518 (1941).

(*8*) Kaplan, H. S., and Rigler, L. G.: Pernicious anemia and carcinoma of the stomach: autopsy studies concerning their interrelationship. Am. J. M. Sc. 209: 339–348 (1945).

(*9*) Black, S., McKibbin, J. M., and Elvehjem, C. A.: Use of sulfaguanidine in nutrition experiments. Proc. Soc. Exper. Biol. & Med. 47: 308–310 (1941).

(*10*) Stewart, H. L., and Jones, B. F.: Pathologic anatomy of chronic ulcerative cecitis: A spontaneous disease of the rat. Arch. Path. 31: 37–54 (1941).

(*11*) MacKenzie, C. G., and MacKenzie, J. B.: Effect of sulfonamides and thioureas on the thyroid gland and basal metabolism. Endocrinology 32: 185–209 (1943).

(*12*) Ashburn, L. K., Daft, F. S., Endicott, K. M., and Sebrell, W. H.: Lesions in rats given sulfaguanidine in purified diets. Pub. Health Rep. 57: 1883–1891 (1943).

(*13*) Gross, P., Axelrod, A. E. and Bosse, M. D.: Lesions in rats fed sulfaguanidine in purified diets. Am. J. M. Sc. 208: 642–660 (1944).

(*14*) Kornberg, A.: Amino acids in the production of granulocytes in rats. J. Biol. Chem. 164: 203–212 (1946).

(*15*) Wells, H. G., Slye, M., and Holmes, H. F.: Comparative pathology of cancer of the alimentary canal: report of cases in mice. Am. J. Cancer 33: 223–238 (1938).

(*16*) Wilson, R. H., de Eds, F., and Cox, A. J.: The toxicity and carcinogenic activity of 2-acetaminofluorene. Cancer Research 1: 595–608 (1941).

(*17*) Bielschowsky, F.: Comparison of the tumors produced by 2-acetyl-amino-fluorene in piebald and Wistar rats. Brit. J. Exper. Path. 27: 135–139 (1946).

(*18*) Lorenz, E., and Stewart, H. L.: Intestinal carcinoma and other lesions in mice following oral administration of 1,2,5,6-dibenzanthracene and 20-methylcholanthrene. J. Nat. Cancer Inst. 1: 17–40 (1940).

(*19*) White, J., and Stewart, H. L.: Intestinal adenocarcinoma and intra-abdominal hemangio-endothelioma in mice ingesting methylcholanthrene. J. Nat. Cancer Inst. 3: 331–347 (1942).

(*20*) Bielschowsky, F.: Distant tumors produced by 2-amino- and 2-acetyl-amino-fluorene. Brit. J. Exper. Path. 25: 1–4 (1944).

(*21*) Stewart, H. L., and Lorenz, E.: Histopathology of induced precancerous lesions of the small intestine of mice. J. Nat. Cancer Inst. 7: 239–268 (1947).

(*22*) Ewing, J.: Neoplastic diseases; a treatise on tumors. W. B. Saunders Co., Philadelphia (1942).

(*23*) Klein, A. J., and Palmer, W. L.: Experimental gastric carcinoma: a critical review with comments on the criteria of induced malignancy. Arch. Path. 29: 814–844 (1940).

TUMORS OF ALIMENTARY TRACT IN MICE FED CARCINOGENIC HYDROCARBONS IN MINERAL-OIL EMULSIONS[1,2]

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INTRODUCTION

Induction of alimentary tract tumors by feeding olive-oil emulsions containing carcinogenic hydrocarbons has been reported (*1*, *2*, *3*). Ingestion of these olive-oil emulsions resulted in a high incidence of carcinoma of the small intestine. In contrast, carcinoma of the forestomach developed rarely and then in only one strain of mice (A). However, when the olive-oil emulsions were made acid stable by the addition of a wetting agent, the dioctyl ester of sodium sulfosuccinic acid (Aerosol OT)[3], squamous carcinoma of the forestomach developed with increasing frequency.

One explanation for this phenomenon is that the stabilized emulsion may form a coating on the inner wall of the forestomach, thus permitting intimate and prolonged contact between the carcinogen and the squamous epithelial lining. If this assumption is correct, it follows that any acid-stable, oil-water emulsion containing a carcinogenic hydrocarbon should have a similar effect. To test this hypothesis an acid-stable, mineral-oil emulsion was selected, since mineral oil is not digested and is slightly, if at all, absorbed in the intestinal tract. It was anticipated that by keeping constant such factors as strain, sex, and age of mice, and the carcinogen employed, but by varying the oil and emulsifying agents, significant differences in the carcinogenic process might be revealed.

The experiments were begun in 1940 and completed in 1941 (*4*), but the analysis and compilation of the data were delayed by the war.

MATERIALS AND METHODS

The hydrocarbons were administered in an aqueous mineral-oil emulsion containing either 0.4 or 0.2 mg. of 20-methylcholanthrene,[4] or 0.16 mg. of 1,2,5,6-dibenzanthracene[5] per cubic centimeter. The maximum melting points of the hydrocarbons were 179.5° C. (corrected) for methylcholanthrene and 267.0° (corrected) for dibenzanthracene. It was difficult to obtain stable mineral-oil emulsions which were acceptable to the mice. The limiting factor was the Aerosol which has an exceedingly bitter taste. When its content was raised above 0.5 percent, the mice refused to drink the emulsion; but less than this amount was insufficient to emulsify 8 percent mineral oil in water. Hence it was necessary to add other emulsifying agents. The final composition of one of the most useful emulsions was as follows: 856 cc. distilled water; 80 cc. light mineral oil;[6] 2 cc. 1N sodium hydroxide; 50 cc. of a 10 percent Aerosol solution in distilled water; 5 gm. cetyl alcohol; 6 gm. monoglycerol stearate; and 1 gm. cholesterol.

The other emulsions listed in tables 1 and 2 differed from the above in having a decreased mineral-oil and Aerosol content, the other constituents remaining unchanged.

In preparing the emulsions, the mineral oil was heated over a water bath and the appropriate amount of hydrocarbon added. When the hydrocarbon was dissolved, the cetyl alcohol, monoglycerol stearate, and cholesterol were added, and heating was continued until a clear solution was obtained. This solution was poured rapidly into distilled water containing the Aerosol solution at room temperature. Emulsification took place immediately. The emulsion was then shaken for a few minutes and stored in the refrigerator, which prevented creaming out for several weeks. By shaking, it could be again homogenized. The feeding bottles containing the emulsions were shaken briefly twice daily to insure a homogeneous emulsion. The procedure of administering the aqueous oil emulsions to mice instead of drinking water was described in a previous communication (*2*).

Records were kept of the weights of all animals and of the quantity of emulsion consumed. The daily consumption of emulsion and of hydrocarbon was calculated for the individual animal by averaging the total intake of the mice in each cage. The consumption per mouse per day fluctuated considerably and tended to rise toward the end of

[1] Received for publication June 18, 1948.

[2] Presented at the meeting of the American Society for Experimental Pathology, Chicago, April 1941.

[3] Hereafter referred to as Aerosol.

[4] Hereafter referred to as methylcholanthrene.

[5] Hereafter referred to as dibenzanthracene.

[6] Clearol, manufactured by Sonnebom Sons Co., New York City.

TABLE 1.—*Consumption of mineral oil emulsions containing methylcholanthrene or dibenzanthracene*

Experimental group	Strain	Number of mice	Age at beginning of experiment	Sex	Emulsion			Duration of experiment	Average quantity ingested per mouse		
					Mineral oil content	Aerosol content	Hydrocarbon content		Emulsion per day	Hydrocarbon per day	Maximum hydrocarbon [1]
			Months		*Percent*	*Percent*	*Milligrams per cubic centimeter*	*Days*	*Cubic centimeters*	*Milligrams*	*Milligrams*
1	A	10	4½	M	8	0.5	Methylcholanthrene 0.4.	206	2.4	1.0	159
2	A	10	4½	M				206	2.4	1.0	168
3	A	10	4½	F				194	2.4	1.0	178
4	C57 brown	10	2½	M				192	3.0	1.2	189
5	do	10	2½	M				186	2.8	1.1	153
6	A backcross	11	5	M				251	2.4	1.0	229
7	do	10	5	F				250	1.9	.7	178
8	I	10	6	M	4	.3	Methylcholanthrene 0.2.	130	2.6	.5	68
9	I	9	7	M				101	3.4	.7	70
10	I	10	6½	M				168	1.4	.3	50
11	I	8	6½	M				188	1.3	.3	50
12	dba	10	6½	F				128	2.7	.5	69
13	A backcross	10	5	M	8	.5	Dibenzanthracene 0.16.	406	2.6	.4	169
14	do	10	5	F				406	2.7	.4	172

[1] A discrepancy between some data of this column with the product of those of columns 9 and 11 is due to the fact that some animals were placed on a water regimen for various periods during the experiment.

the experiment, possibly because of greater longevity among the heavier consumers, or perhaps because surviving mice became better adjusted to the emulsions and gradually consumed more. Tables 1 and 2 give the pertinent data.

Carcinoma of the forestomach was frequently detected clinically as a palpable abdominal mass or indirectly by irreversible weight changes. Animals which appeared to be dying or in which the presence of tumor was suspected were killed with ether to obviate post-mortem changes. Only a small number of animals died during the experiment. The autopsy procedure and pathologic techniques employed have been described (*1*, *3*).

Control animals (table 2) were kept for each experimental group. One control group received a mineral-oil emulsion without the hydrocarbon; the second group, an aqueous solution of 0.5 percent of Aerosol (with the exception of group 11 which received only 0.15 percent); the third group (strains A and C57 brown), distilled water. Only those control groups of strain I and dba mice which survived 100 days or more are listed in table 2. Since mice of these strains did so poorly on the experimental emulsions, no attempt was made to set up additional control groups.

Strains A, C57 brown, and A backcross mice were purchased from the Roscoe B. Jackson Labo-

TABLE 2.—*Fluid consumption of control mice*

Group	Strain	Number of mice	Age at beginning of experiment	Sex	Drinking fluid	Duration of experiment	Average quantity any single mouse ingested per day
			Months			*Days*	*Cubic centimeters*
1	A	10	4½	F	Mineral-oil emulsion, 8 percent mineral oil.	210	2.6
2	A	10	4½	F	Aerosol solution in water, 0.5 percent Aerosol.	210	2.4
3	A	10	4½	F	Distilled water	210	5.0
4	C57 brown	10	2½	M	Mineral-oil emulsion, 8 percent mineral oil.	206	3.4
5	do	10	2½	M	Aerosol solution in water, 0.5 percent Aerosol.	206	2.5
6	do	10	2½	M	Distilled water	209	7.5
7	A backcross	6	5	M	Mineral-oil emulsion, 8 percent mineral oil.	385	3.8
8	do	10	3½	M	Aerosol solution in water, 0.5 percent Aerosol.	396	2.8
9	I	8	6	M	Mineral-oil emulsion, 4 percent mineral oil.	143	3.5
10	I	7	7½	M	do	187	2.0
11	I	4	10	F	Aerosol solution, 0.15 percent Aerosol	175	2.2
12	dba	10	7	F	Aerosol solution, 0.5 percent Aerosol	129	2.0

ratory, Bar Harbor, Maine. Strains I and dba mice were obtained from the stock of this Institute. The strain I mice of experimental groups 10 and 11, together with the controls, received a soft diet, consisting of 10 percent casein, 10 percent yeast, 40 percent starch, 20 percent Crisco, 2 percent cod-liver oil, 14 percent sucrose, and 4 percent inorganic salts. Mice of all other groups were given Purina dog chow. Food and fluid intake were not restricted, but emulsion replaced the drinking water as indicated in tables 1 and 2.

RESULTS

It was difficult to keep the experimental animals alive, and it was often necessary to place them either individually or in groups on a water regimen from time to time when rapid weight loss occurred. As shown in tables 1 and 2, the daily intake of emulsion or of Aerosol solution by strains A and C57 brown mice was approximately only half of that of water. The 0.4-mg. methylcholanthrene-mineral oil emulsion was just about as toxic as its olive oil counterpart used in previous experiments (*1*). It was especially toxic in strains I and dba mice. In spite of a reduction of methylcholanthrene, mineral oil, and Aerosol content, mice of several groups died within a few weeks. The majority of the animals of groups 8 and 9 also died early in the experiment; only the animals of groups 10 and 11, which were given the soft diet, survived for longer periods. The total estimated intake of methylcholanthrene by animals of these two strains surviving 100 days or more was between 50 and 70 mg. per mouse or only one-third that of the other strains.

The changes observed in the forestomach of mice ingesting mineral-oil emulsions containing methylcholanthrene and dibenzanthracene have been described briefly (*4*). Data on the type, location, and incidence of these changes, and the amounts of hydrocarbon necessary to induce them in different strains of mice are here presented.

Although no carcinomas of the esophagus developed, the dyskeratotic areas were indistinguishable from precancerous lesions of the forestomach. The forestomach showed acanthosis, hyperkeratosis, papillomas, focal and diffuse dyskeratosis (precancerous lesions), and squamous-cell carcinoma. In the small intestine there were only a few precancerous lesions and carcinoma. In addition, tumors in the lungs of susceptible strains and a single hemangio-endothelioma of the small intestine were observed. There were a few cases of leukoplakia of the tongue in animals of strains C57 brown and A backcross. A notable feature was the pronounced vacuolization of hepatic cells in strain I mice.

Table 3 shows that single or multiple, solitary or massive, squamous-cell carcinoma of the forestomach developed in 57 of 138 experimental mice. Precancerous changes were observed in most of these 57 mice and in 18 additional animals. All these 57 carcinomas occurred in 91 animals of the highly susceptible strains A, C57 brown, and A backcross mice.

Every lesion diagnosed as carcinoma of the forestomach showed invasion through all coats of the viscus and the presence of a neoplastic nodule on the peritoneum. Any dyskeratotic lesion showing more limited infiltration of the gastric wall was classified as precancerous. The pathologic features of the carcinomas and precancerous lesions are described in another paper.[7] Suffice it to say here that the carcinomas showed a pronounced ability to invade adjacent structures and organs and to metastasize. Of the 57 mice with carcinomas of the forestomach, 31 showed infiltration or metastases, and 10 of the 31 showed both infiltration and metastases involving the peritoneum, mesentery, omenta, abdominal blood vessels, pancreas, liver, spleen, diaphragm, mesenteric lymph nodes, kidney, and abdominal and thoracic wall. Four tumors were serially transplanted subcutaneously to mice of the strain of origin, and all grew.

In the most susceptible strains (A, C57 brown, and A backcross hybrids), the first forestomach tumors induced with methylcholanthrene were observed between 83 and 130 days, corresponding to an average ingestion of hydrocarbon of 70 to 103 mg. Considering that the tumors may have existed for a varying length of time before becoming palpable, the data indicate that these mice are of approximately equal susceptibility. No tumors were observed in strains I and dba. This would seem to be attributable in part to the failure of these mice to ingest sufficient quantities of methylcholanthrene. The forestomach of the dba mice

[7] STEWART, H. L., and LORENZ, E.: Publication pending.

TABLE 3.—*Tumors and other lesions of the forestomach and esophagus induced by methylcholanthrene or dibenzanthracene mineral oil emulsion*

Group	Strain	Number of mice	Sex	Emulsions			Average total intake of hydrocarbon until first tumor appeared [1]	Duration of experiment until first tumor appeared	Mice with single or multiple squamous-cell carcinoma of forestomach	Mice with malignant dyskeratosis only of forestomach [2]	Mice with benign lesions of forestomach only	Invasion of adjacent organs and structures	Metastases	Malignant dyskeratosis of esophagus
				Mineral oil	Aerosol	Hydrocarbon								
				Percent	*Percent*	*Milligrams per cubic centimeter*	*Milligrams*	*Days*	*Number*	*Number*	*Number*	*Number*	*Number*	
1	A	10	M	8	0.5	Methylcholanthrene 0.4.	70	90	8	2	None	4	3	None
2	A	10	M				90	90	7	2	1	1	1	None
3	A	10	F				91	94	7	2	1	5	2	1
4	C57 brown	10	M				163	86	8	2	None	1	6	2
5	do	10	M				83	83	10	6	None	4	1	1
6	do	11	M				94	94	6	4	1	3	2	None
7	A backcross	10	F				91	130	9	None	1	5	3	1
8	do	10	F						None	None	3	None	None	None
9	I	10	M	4	.3	Methylcholanthrene 0.2.			None	None	1	None	None	None
10	I	9	M						None	None	6	None	None	None
11	I	10	M						None	None	None	None	None	None
12	I	8	M				154	100	None	1	None	None	None	None
13	dba	10	F			Dibenzanthracene 0.16.	166	166	None	None	5	None	None	None
14	A backcross	10	M	8	.5		166	166	1	1	8	None	None	None
	do	10	F				152	379	2	4	3	None	None	None

[1] See footnote 1, table 1. [2] Precancerous lesions.

nevertheless would appear to be susceptible to cancer induction since in one instance a lesion developed which was indistinguishable from the precancerous lesions of the mice of the other strains. With dibenzanthracene only two squamous-cell carcinomas of the forestomach developed in strain A backcross hybrid; the first carcinoma appeared after 379 days, when the average intake of carcinogen per mouse was 152 mg., indicating the lower potency of this compound as compared with methylcholanthrene.

Precancerous lesions were usually multiple and were observed either alone or in association with carcinoma. As would be expected, they appeared before the first carcinoma was observed. These precancerous lesions and their evolution into carcinoma are described in another paper.[8] Acanthosis, hyperkeratosis, and papillomas were observed in both the experimental and in many of those control animals which were given either mineral oil emulsion or Aerosol solution (table 4).

The glandular mucosa of the pyloric chamber of the stomach proved completely refractory to the induction of tumors by the oral administration of carcinogenic hydrocarbons in mineral-oil emulsions, thus confirming previous experience when the hydrocarbons were given in olive oil. It is of special interest to note that the spontaneous adenomatous lesion of the glandular stomach of I and dba mice was not influenced by hydrocarbon administration (*5*). In both the experimental and control animals of these strains, the extent of this gastric glandular lesion was within the limits observed in untreated control animals of comparable age.

[8] See footnote 7.

DISCUSSION

The present and previous studies of experimental gastro-intestinal cancer have brought out certain interesting facts. When the carcinogenic hydrocarbons, methycholanthrene and dibenzanthracene, are administered in oil-water emulsions the two chief sites at which tumors are induced are the forestomach and the small intestine. The forestomach tumors arise from the squamous epithelial lining and the small intestinal tumors from the glandular mucosa. Methylcholanthrene is more potent than dibenzanthracene in inducing tumors in either of the foregoing locations, which is in accordance with reports of the comparative carcinogenic potency of these compounds when tested upon other tissues such as skin or subcutaneous connective tissue. There is also a variation in the number of tumors induced depending upon the strain of mouse employed. The most susceptible are strains A, C57 brown, and A backcross mice in this order. Strains I and the dba mice were refractory under these experimental conditions. Most striking, perhaps, is the observation that carcinoma of the glandular stomach has not been induced when the carcinogens are administered by the oral route, as in these experiments.

A notable difference was observed in the induction of carcinoma of the forestomach by altering the nature of the olive-oil emulsion. When the standard olive-oil emulsion was employed as the vehicle, the carcinogenic effect of the hydrocarbons

TABLE 4.—*Lesions observed in control mice*

Group	Strain	Number of mice	Sex	Drinking fluid	Number of benign lesions of forestomach	Other observations
1	A	10	F	Mineral-oil emulsion 8 percent mineral oil.	6 (pronounced)	
2	A	10	F	Aerosol solution 0.5 percent Aerosol.	2	
3	A	10	F	Distilled water	None	
4	C57 brown	10	M	Mineral-oil emulsion 8 percent mineral oil.	10	4 lingual leukoplakia.
5	do	10	M	Aerosol solution 0.5 percent Aerosol.	3	
6	do	10	M	Distilled water	None	
7	A backcross	6	M	Mineral-oil emulsion 8 percent mineral oil.	4 (pronounced)	1 lingual leukoplakia.
8	do	10	M	Aerosol solution 0.5 percent Aerosol.	5	Do.
9	I	8	M	Mineral-oil emulsion 4 percent mineral oil.	3	
10	I	7	M		6	
11	I	4	F	Aerosol solution 0.15 percent Aerosol.	2	
12	dba	10	F	Aerosol solution 0.5 percent Aerosol.	None	

was manifested in the small intestine, and very few forestomach tumors were induced. When, however, the olive-oil emulsion was stabilized by the addition of Aerosol, squamous-cell carcinoma of the forestomach was induced in high incidence. This striking difference in the carcinogenic effect on the forestomach mucosa of these two olive-oil emulsions has been explained on the assumption that the standard nonstabilized emulsion will break at the pH of the forestomach with the formation of oil globules, thus preventing intimate contact of the carcinogen with the mucosa. The stabilized emulsion, on the other hand, probably coats the mucosa of the forestomach, providing a more or less constant exposure to the carcinogen. It can be assumed that in experimental tumor induction, the carcinogenic hydrocarbon must be in contact with the tissue of origin for a certain minimum period in order to induce the neoplasm and that intermittent exposure increases the induction time.

Like the stabilized olive-oil emulsion, the mineral-oil emulsion used in the present experiments possesses the ability to induce carcinoma of the forestomach in a large percentage of the mice. As it is also acid stable, we have assumed that its carcinogenic action on the mucosa of the forestomach can be explained by the same hypothesis. There is no evidence that Aerosol has a cocarcinogenic action. Moreover, in an experiment[9] in which the Aerosol was replaced by sulfonated castor oil or by sodium taurocholate, tumors of the forestomach were induced in high incidence.

The mineral-oil emulsion seems to be more potent carcinogenically than the stabilized olive-oil emulsion so far as the forestomach mucosa is concerned. The first tumors to appear in strain A backcross hybrids ingesting the mineral-oil emulsion of methylcholanthrene required 94 days and 94 mg. of carcinogen in the male and 130 days and 91 mg. of carcinogen in the female. With the stabilized olive-oil emulsion of methylcholanthrene, the first tumor required 168 days and 168 mg. of the carcinogen in the male and 196 days and 137 mg. of the carcinogen in the female. Moreover, malignant dyskeratotic lesions of the esophagus developed only in those mice ingesting the mineral-oil emulsion, even though the exposure to the emulsion was intermittent. Two factors may contribute to the apparently greater carcinogenic effect of the mineral-oil emulsions: (1) Other emulsifying agents were employed in these emulsions in addition to the Aerosol thereby increasing their wetting power; (2) the viscosity of the mineral-oil emulsion is considerably higher than that of the stabilized olive-oil emulsion. Both factors would cause the emulsion to adhere better to surfaces. However, it is felt that the viscosity of the emulsion is the more important of the two factors. There is the additional possibility to be considered that the animals receiving the olive-oil emulsion are actually on a high fat diet which itself may influence tumor induction of the forestomach.

Like the olive-oil emulsions, the mineral-oil emulsions did not induce any tumors of the glandular stomach. The previous explanation (*3*) that due to the cleansing action of the secretions there is probably failure to coat the mucosa with the emulsion still seems valid. Since the glandular mucosa is constantly bathed by mucus, the carcinogen may never actually come in contact with the epithelial cells or the underlying stroma. On the other hand, gastric adenocarcinoma is readily induced in mice by injection of methylcholanthrene into the walls of the stomach (*6*). A series of other unpublished experiments in which different emulsions or suspensions containing carcinogenic hydrocarbons were given orally to mice likewise failed to induce tumors of the glandular stomach. It seems reasonable, therefore, to assume that the gastric glandular mucosa of the mouse is refractory to carcinogenesis if the carcinogenic agent (polycyclic hydrocarbon) is contained in the food or drinking fluid. Since, however, the glandular mucosa of the mouse is susceptible to cancer induction, the suggestion is made that an intrinsic factor contained in the body fluids and introduced interstitially into the viscus may play an important role in the induction of gastric cancer. Whether one is justified in translating this experience from mouse to man remains undetermined.

When the mineral-oil emulsion enters the small intestine, only the organic constituents of the emulsion are digested to any extent and of these only the cetyl alcohol and the glycerol monostearate carry along with them small amounts of carci-

[9] LORENZ, E.: To be published.

nogen. The bulk of the carcinogen remains dissolved in the mineral oil. In discussing the high incidence of intestinal tumors with the olive oil emulsion, it was stated (*2*) that during and after digestion of the olive oil the hydrocarbon is precipitated out and thus comes in intimate contact with the intestinal mucosa through which it is absorbed. Such an assumption is favored by the induction of multiple lung tumors in the majority of susceptible animals (strain A, strain A backcross hybrids) and of sarcoma and hemangioendothelioma of the mesentery and retroperitoneal tissues along the route of absorption; and evidence of systemic effects such as severe atrophy of the genital organs, the spleen, lymph nodes, and hematopoietic tissues, previously observed (*1*, *2*). On the other hand, with the mineral-oil emulsion, which does not undergo digestion to any great extent, the carcinogenic effects on the small intestine and other tissues as well as the toxic effects enumerated should be greatly diminished. This opinion is supported in the present experiments in which the amounts of methylcholanthrene ingested were sufficient to induce carcinoma of the small intestine if it had been administered in olive oil. With the mineral-oil emulsion, no carcinoma of the small intestine was observed in 50 mice of strains A and A backcross hybrids while only 5 of 20 C57 brown mice developed this tumor (table 5). With the methylcholanthrene-olive oil emulsion, intestinal tumors occurred in 16 of 30 strain A mice, 7 of 20 strain A backcross mice, and in 11 of 25 C57 brown mice. No retroperitoneal sarcomas were observed, and only a single intestinal hemangio-endothelioma was observed in the mice given mineral-oil emulsions. The number of animals with lung tumors and the number of tumors per mouse in susceptible strains were also considerably reduced as compared with the olive-oil emulsion experiments. Atrophic effects on genital organs, bone marrow, spleen, and lymph nodes were also considerably reduced in the animals given the mineral-oil emulsion, indicating decreased absorption of the carcinogen from the mineral oil. This favors the previously expressed hypothesis (*2*) that the effect is direct (local) rather than indirect (systemic) since the peritoneal and retroperitoneal tumors develop along the route of absorption of the carcinogen from the intestinal tract. These findings suggest further that oral ingestion of hypothetical carcinogens and their subsequent absorption from the intestinal tract may lead to the induction of neoplasm at sites other than the intestinal tract.

No tumors were induced in the large intestine although a considerable amount of carcinogen dissolved in mineral oil must have reached the large intestine. In an earlier experiment (*1*) it was shown by ultraviolet absorption spectrometry of feces of mice given dibenzanthracene-olive oil emulsions that the carcinogen was converted into a new noncarcinogenic compound either by the intestinal flora or by some other unknown mechanism. Since ultraviolet absorption spectra of

TABLE 5.—*Small intestine and pulmonary tumors induced by methylcholanthrene and dibenzanthracene mineral oil emulsions*

Group	Strain	Sex	Number of mice	Emulsions			Duration of experiment when first intestinal lesion was observed	Mice with carcinoma of small intestine	Mice with precancerous lesions of small intestine only	Mice with pulmonary tumors
				Mineral oil	Aerosol	Hydrocarbon				
				Percent	*Percent*	*Milligrams per cubic centimeter*	*Days*	*Number*	*Number*	*Number*
1	A	M	10	8	0.5	0.4 (metylcholanthrene).	[1] 90	None	1	4
2	A	M	10				[1] 94	None	2	3
3	A	F	10					None	None	5
4	C57 brown	M	10				109	2	3	1
5	do	M	10				122	3	1	None
6	A backcross	M	11				[1] 251	None	1	3
7	do	F	10				[1] 251	None	1	4
8	I	M	10	4	.3	0.2 (methylcholanthrene)		None	None	None
9	I	M	9				[1] 101	None	1	None
10	I	M	10					None	None	None
11	I	M	8				[1] 180	None	2	2
12	dba	F	10					None	None	None
13	A backcross	M	10	8	.5	0.16 (dibenzanthracene)		None	None	4
14	do	F	10					None	None	4

[1] Precancerous lesions.

feces were not recorded in the present study, we do not know whether or not the carcinogen was changed in the large intestine or whether the large intestine is refractory to tumor induction.

SUMMARY

Aqueous mineral-oil emulsions containing either methylcholanthrene or dibenzanthracene were given instead of drinking water to four inbred strains of mice (A, C57 brown, dba, I) and strain A backcross hybrids.

Squamous-cell carcinoma of the forestomach was induced by methylcholanthrene in a large number of mice of strains A and C57 brown and strain A backcross hybrids, but dibenzanthracene induced few such tumors.

Malignant dyskeratosis of the forestomach was induced with methylcholanthrene and dibenzanthracene in animals of all strains but strain I.

Few carcinomas and precancerous lesions were induced in the small intestine in contrast with previous experiments in which olive-oil emulsions were employed.

Factors governing the varied carcinogenic response of the gastro-intestinal tract to different emulsions containing carcinogenic compounds are discussed.

REFERENCES

(*1*) Lorenz, E., and Stewart, H. L.: Intestinal carcinoma and other lesions in mice following oral administration of 1,2,5,6-dibenzanthracene and 20-methylcholanthrene. J. Nat. Cancer Inst. 1: 17–40 (1940).

(*2*) Lorenz, E., and Stewart, H. L.: Tumors of the alimentary tract induced in mice by feeding olive oil emulsions containing carcinogenic hydrocarbons. J. Nat. Cancer Inst. 7: 227–238 (1947).

(*3*) Stewart, H. L., and Lorenz, E.: Histopathology of induced precancerous lesions of the small intestine of mice. J. Nat. Cancer Inst. 7: 239–268 (1947).

(*4*) Lorenz, E., and Stewart, H. L.: Squamous-cell carcinoma and other lesions of the forestomach in mice following oral administration of 20-methylcholanthrene and 1,2,5,6-dibenzanthracene (preliminary report). J. Nat. Cancer Inst. 1: 273–276 (1940).

(*5*) Stewart, H. L., and Andervont, H. B.: Pathological observations on adenomatous lesions of stomach in mice of strain I. Arch. Path. 26: 1009–1022 (1938).

(*6*) Stewart, H. L., and Lorenz, E.: Adenocarcinoma of the pyloric stomach and other gastric neoplasms in mice induced with carcinogenic hydrocarbons. J. Nat. Cancer Inst. 3: 175–189 (1942).

IMPROVEMENT IN THE METHOD FOR THE HISTOCHEMICAL DEMONSTRATION OF ALKALINE PHOSPHATASE AND ITS USE IN A STUDY OF NORMAL AND NEOPLASTIC TISSUES[1,2]

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INTRODUCTION

An ingenious procedure for demonstrating in tissue the site of activity of alkaline phosphatase was introduced in 1944 by Menten, Junge, and Green (*1*). Their method consists in incubating tissue sections in a solution of calcium beta naphthyl phosphate (I) and alpha naphthyl diazonium chloride (III) buffered to pH 9.4. The beta naphthol (II) liberated by phosphatase activity immediately couples with the diazonium salt to form an insoluble, purplish-red pigment (VI), which precipitates at the site of its formation. The method requires diazotization of naphthylamine in the cold immediately before use followed by careful adjustment of the pH. This intricate procedure probably has discouraged routine use of an otherwise satisfactory histochemical method (chart 1).

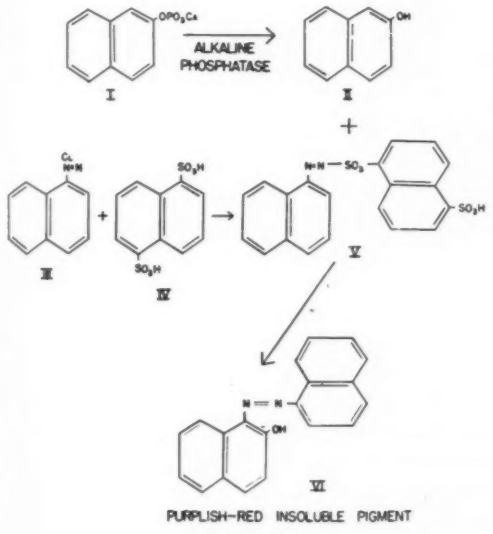

CHART 1.

We have overcome this objection to the method by preparing a stabilized alpha naphthyl diazonium salt (V) by the addition of naphthalene-1, 5-disulfonic acid (IV). The crystalline salt can be stored in the cold for months. The method is thus simplified since it disposes of the need for carrying out the diazotization procedure on each occasion before staining the tissue sections and merely requires the addition of two powders (I and V) to a cold buffer at pH 9.4 just before the tissue sections are immersed.

The preparation of the reagents and the use of this method in demonstrating alkaline phosphatase in some normal and neoplastic tissues form the basis of this paper.

EXPERIMENTAL PROCEDURE

CALCIUM BETA NAPHTHYL PHOSPHATE (I)

The following preparation was found to be more convenient than the method (*1*) given previously: Beta naphthol (14.4 gm.) was dissolved in 15 cc. of dry pyridine[4] and added in portions to phosphorous oxychloride (15.3 gm.) solution in 10 cc. of dry pyridine. Heat was developed by the reaction, and a white precipitate separated as a sludge. After standing at room temperature for 10 minutes, the reaction mixture was cooled and added in portions to a solution of calcium acetate (30 gm.) in 200 cc. of water and 100 gm. of ice. An oily material separated, which slowly dissolved and came out as a white precipitate. Alcohol (150 cc.) was then added, and the white precipitate was collected with the aid of suction, washed with 95 percent alcohol, ether, and dried; yield 18.8 gm. (72 percent).

No test for naphthol was obtained on suspending the powder in a solution of a diazonium compound. Storage for 1 year did not result in the appearance of naphthol. Boiling with mineral acid or incubation in blood plasma produced naphthol.

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[2] This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service, and in part by a research grant from the American Cancer Society (Massachusetts division).

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[4] Reagent pyridine contains enough water to decompose the phosphorous oxychloride extensively. It was dried by refluxing with anydrous barium carbonate for several days, distilled, and stored with sodium hydroxide pellets.

Calcium Alpha Naphthyl Phosphate

The phosphoric ester of alpha naphthol was prepared in the same manner as the beta isomer. Hydrolysis of the chloro compound in calcium acetate proceeded slower than with the beta isomer; yield 17 gm. (65 percent).

Alpha Naphthyl Diazonium Naphthalene-1,5-Disulfonate (V) (*2*)

Alpha naphthylamine hydrochloride (18 gm.) was dissolved in boiling water (150 cc.) and cooled. Concentrated hydrochloric acid (12 cc.) was added to precipitate the hydrochloride as a sludge. The mixture was then cooled to 10° C. A solution of sodium nitrite (7.5 gm.) in water (25 cc.) was run in slowly with stirring at such a rate that the temperature did not rise above 12°. Ten minutes after all the nitrite had been added, the dark solution was filtered rapidly with suction. Naphthalene-1,5-disulfonic acid (IV) (30 gm.) was added, and the mixture was stirred vigorously. The sulfonic acid dissolved, and a heavy sludge of greenish-tan crystals separated. This was collected with the aid of suction, washed with alcohol, ether, and dried at 37°; yield, 33 gm. (67 percent).

The material should be kept cold and protected from light. Eight months of storage at 4° C. did not result in deterioration. After several weeks at room temperature, the powder darkened and contained water-insoluble tarry material. Decomposition took place rapidly in solution (1 hour), particularly at pH above 7 and at elevated temperature (37°). The pigment produced with beta naphthol was purplish-red, and with alpha naphthol was brown.

Other Stabilized Diazonium Compounds

Diazonium compounds prepared from ortho dianisidine and 2-amino-4-chloroanisole and stabilized in powder form are available commercially as "Naphthanil Diazo Blue B"[5] which includes 4.8 percent zinc chloride and "Naphthanil Diazo Red RC"[6] which contains 5 percent zinc chloride and 15 percent aluminum sulfate. The pigments which are produced by coupling with beta naphthol are indicated in the commercial designation. "Diazo Blue B" is a tetrazonium compound and therefore couples with two molecules of naphthol in the staining reaction.

[5] [6] Provided through the courtesy of Dr. E. R. Laughlin, duPont de Nemours & Co., Boston.

Fixation of Tissues

Fresh tissue was fixed on 95 percent alcohol or in cold acetone for 12–24 hours. Alcohol produced less tissue shrinkage than did acetone. But acetone was preferred when the blocks of tissue were to be used for demonstrating acid phosphatase. The effect of length of time of fixation in these solvents on the destruction of alkaline and acid phosphatase was determined recently (*3*).

Paraffin embedding was accomplished via absolute alcohol or acetone (three changes in 24 hours), cedar-wood oil (overnight), xylol (two changes in 1 hour), and paraffin (three changes in 3 hours). Low-melting paraffin (m. p. 52°–54° C.) was utilized, and embedding was performed at an incubator temperature of 56°–58°. Sections were cut 6μ in thickness.

Demonstration of Alkaline Phosphatase

Paraffin-embedded sections fixed to slides with albumin were carried through xylol and alcohol (or acetone) to water and incubated at 10° C. in the following freshly prepared solution: Calcium beta naphthyl phosphate (I), 50 mg.; alpha naphthyl diazonium naphthalene-1, 5-disulfonate (V), 50 mg.; magnesium sulfate (1 percent), 1 cc.; barbital buffer[7] pH 9.4, 15 cc.; and water, 84 cc.

To insure solution, both powders were ground with a little cold water in a mortar. Solution of the phosphate was not complete, and occasional agitation assured further solution as the phosphate was hydrolyzed. Dye deposition was noted in phosphatase-rich tissues within 1 minute, and maximum staining was obtained in 15–20 minutes. The solution darkened as the diazonium salt decomposed. By using the diazonium salt in low concentration, by conducting the reaction at a low temperature, and by keeping the period of incubation short, coupling of the diazonium compound with the tissue protein to give a diffuse, pale-brown color to the section was kept at a minimum. Before mounting, the slides were washed with water for a few minutes.

[7] Prepared according to Michaelis (*4*) by bringing 10.3 gm. of sodium diethyl barbiturate to 500 cc. with distilled water. To obtain buffer at pH 9.4, 0.25 cc. of 0.1M HCl was added to 9.75 cc. of the barbital solution. Other buffers may be used.

In some sections, the nuclei were lightly counterstained with a dilute, aqueous solution of alum hematoxylin (0.25 percent) for 20 to 30 seconds. The stability of the azo dye in acid makes possible decalcification of bone after the stain has been developed. Frozen sections can then be made (fig. 1, *D*). The photomicrographs (figs. 1–10) were made with panchromatic film through a green filter.

Because of the solubility of the azo dye in organic solvents, permanent mounts of the sections were made in aqueous medium. Kaiser's medium [8] sets to a gel at room temperature. The cover slips seal after several days of drying.

Calcium alpha naphthyl phosphate was found to be unsuitable as a substrate for alkaline phosphatase, as indicated by relatively weak staining under conditions optimal for the beta substrate. However, the alpha substrate gave results superior to the beta product with acid phosphatase. This observation formed the basis for the development of a method for acid phosphatase (to be published).

RESULTS

Normal Tissues

Since the substrate used in this histochemical method for alkaline phosphatase does not occur in nature, it was necessary to compare the distribution of phosphatase by this method with the distribution found by others (*6–9*) with Gomori's method (*6*), which utilizes naturally occurring phosphoric esters as substrate. No significant differences were found. The tissues richest in alkaline phosphatase are illustrated in figure 1. They are kidney (cat) (fig. 1, *A*); duodenum (dog) (fig. 1, *B*), and bone (costochondral junction in the mouse (fig. 1, *C*), and in man (fig. 1, *D*).

The endothelium of blood vessels under 80μ in diameter was rich in phosphatase in all tissues. The blood vessels of the central nervous system were particularly rich in phosphatase (fig. 2, *A*), as observed previously (*10*). Similar richness of staining of the vascular endothelium was seen only in gall bladder and striated muscle. In sharp contrast, the vascular endothelium of neoplastic tissue was deficient in alkaline phosphatase.

[8] The formula, as given by Mallory (*5, p. 100*) is: Gelatin, 40 gm.; water, 210 cc.; glycerin, 250 cc.; and phenol, 5 gm.

Contrary to observations of some (*6–8, 11*), scattered stomach glands showed phosphatase activity. In the cat, these cells were distributed along the epithelium of the glandular crypts; in man, they were concentrated on the surface epithelium and distal half of the pits (fig. 2, *B*); and in the dog, they were visible only on the surface epithelium.

Human Tumors

Of the tumors examined, alkaline phosphatase was found regularly only in ovarian carcinoma, Grawitz tumor (hypernephroma), osteogenic sarcoma, and benign breast tumors. Table 1 summarizes the results with 129 tumors (95 neoplastic and 34 hyperplastic). The tumors [9] in most cases are listed under the tissue of origin. The alkaline phosphatase content was roughly estimated from the intensity of stain obtained under constant conditions of fixation, embedding, pH, temperature, time of incubation with substrate, and concentration of reagents. In separate columns of table 1, the phosphatase content of tumor cells, tumor stromal cells, and vascular endothelium of tumor is compared with the phosphatase content of the corresponding cells in the original organ (except in the case of Krukenberg tumors). In most cases, the comparisons were made in the same section. Estimations for each tumor were obtained from four to six slides. Each organ is considered separately in the following sections.

Stomach

Of 15 tumors, 4 showed alkaline phosphatase activity within the tumor cells. Three of these were adenocarcinomas (3 of 6), and in these only occasional glands contained scattered deposits beneath the cuticular borders of the cells. The other tumor was a papilloma in which several glands exhibited deep stain in the Golgi region of the cells and in the luminal cuticular border (fig. 3, *A*). The papilloma cells were strongly eosinophilic as compared with the surrounding mucin-containing normal cells. Blood vessel walls containing phosphatase were seen in both the tumor and the normal stomach. Four undifferentiated carcinomas, one colloid carcinoma, one carcinoma in situ, one lymphoblastoma, and one neurofibroma

[9] The pathologic diagnosis was made from eosin-hematoxylin-stained sections by Drs. Monroe Schlesinger and Leopold Reiner, Department of Pathology, Beth Israel Hospital. Photomicrographs were made by Percy Hemlow and Leo Goodman.

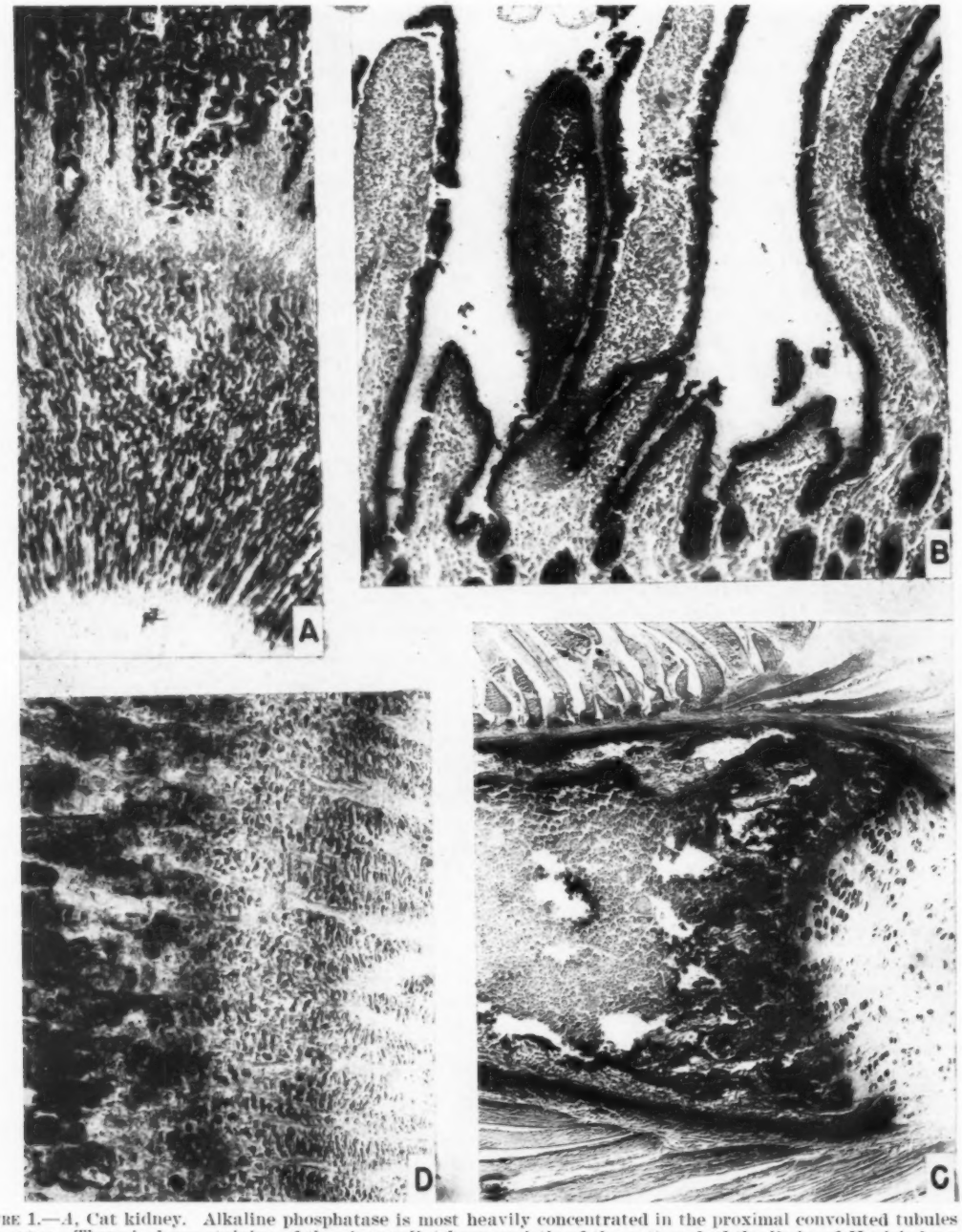

FIGURE 1.—*A*, Cat kidney. Alkaline phosphatase is most heavily concentrated in the proximal convoluted tubules of the cortex. There is dense staining of the glomeruli (characteristic of the cat) and of the limbs of Henle's loop. Note zonal arrangement of the tissue containing phosphatase. × 20. *B*, Dog duodenum. Alkaline phosphatase is concentrated at the cuticular border and Golgi region of the mucosal cells. Cell nuclei are counterstained lightly with hematoxylin. × 90. *C*, Mouse rib. Alkaline phophatase is concentrated at the costochondral junction and in the subperiosteal region. Blood vessel in skeletal muscle at lower left corner. × 100. *D*, Human costochondral junction. Phosphatase activity is greatest in the zone of calcification. A 3-mm.-thick block of the bone was stained for alkaline phosphatase and was then decalcified with 5 percent nitric acid for 2 days. Frozen section was 16μ thick. × 110.

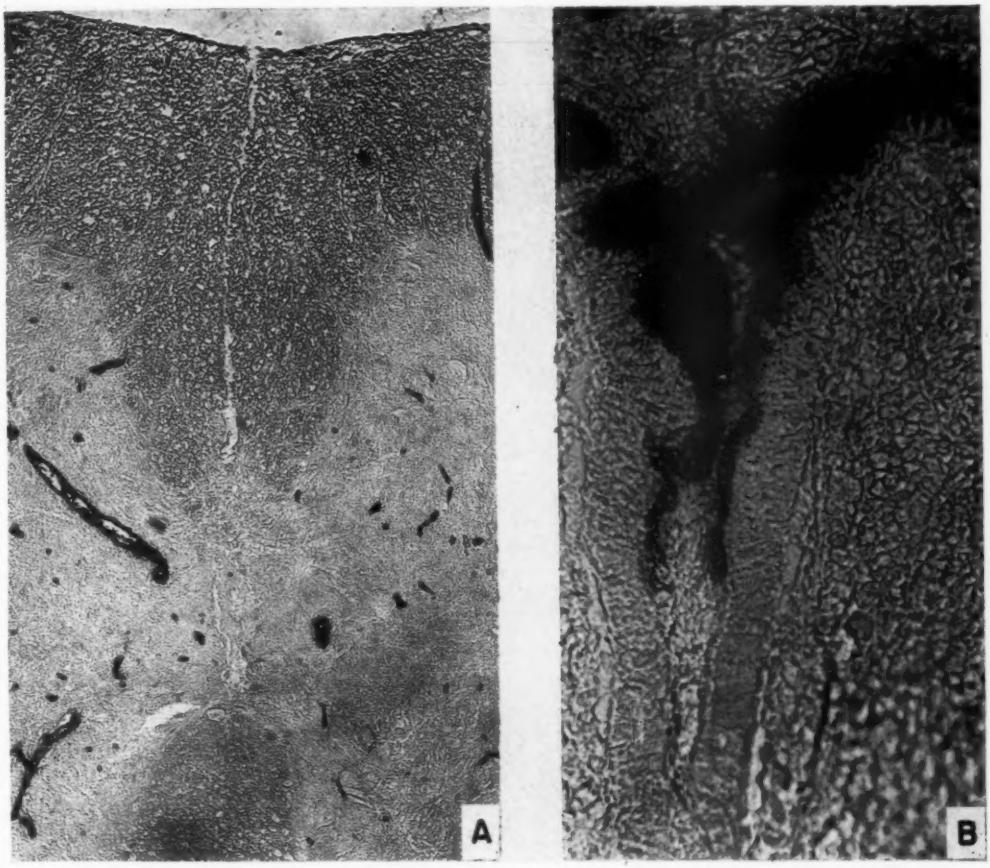

FIGURE 2.—*A*, Mouse spinal cord. Heavy staining due to alkaline phosphatase outlines the course of the blood vessel. × 110. *B*, Human stomach. Alkaline phosphatase is concentrated at the luminal end of a glandular pit. Oblique section. × 500.

were devoid of phosphatase. With the exception of 1 undifferentiated carcinoma, the anaplastic tumors were devoid of phosphatase-containing blood vessels. The blood vessels of the more differentiated tumors were deficient in phosphatase-containing blood vessels, as compared with those of the normal stomach.

JEJUNUM

One adenocarcinoma examined was devoid of phosphatase.

COLON-RECTUM

No phosphatase was seen in the 19 tumors studied, except in vascular endothelium (fig. 3, *C*). Only in a papilloma was the intensity of phosphatase in the blood vessels equal to that in normal colon.

BREAST

The undifferentiated carcinomas, an adenocarcinoma, and a fibrosarcoma failed to show phosphatase activity in the tumor cells. The blood-vessel endothelium gave a weak reaction, as compared with normal breast tissue, with the exception of one carcinoma. Phosphatase was seen in fibrous stroma of normal breast tissue but not in the stroma of any of these tumors.

In seven specimens of fibrocystic disease and in areas of fibrocystic disease in some of the breasts with carcinoma, moderate concentration of alkaline phosphatase was noted at the basement mem-

TABLE 1.—*Comparison of alkaline phosphatase distribution in human normal and tumor tissue*

Type of tumor	Number of tumors	Alkaline phosphatase content [1]						Average age of patients
		Parenchymal cells		Stromal cells		Blood vessels		
		Normal [2]	Tumor	Normal	Tumor	Normal	Tumor	
								Years
Stomach:								
Adenocarcinoma	6	±	±	0	0	+++	+	67
Undifferentiated carcinoma	4	±	0	0	0	+++	+	61
Colloid carcinoma	1	±	0	0	0	+++	++	67
Carcinoma in situ	1	±	0	0	0	+++	+	61
Lymphoblastoma	1	0	0	0	0	+++	0	51
Papilloma	1	±	±	0	0	+++	++++	55
Neurofibroma	1	0	0	0	0	+++	+++	55
Jejunum:								
Adenocarcinoma	1	±	0	±	0	+++	++	49
Colon-rectum:								
Adenocarcinoma	13	0	0	0	0	+++	+	61
Colloid carcinoma	2	0	0	0	0	+++	+	61
Papillary carcinoma	3	0	0	0	0	++	+	63
Papilloma	1	0	0	0	0	+++	++	68
Breast:								
Undifferentiated carcinoma	10	0	0	+	0	++	±	49
Adenocarcinoma	1	0	0	+	0	++	±	54
Fibrosarcoma	1	0	0	+	0	++	+	45
Fibrocystic disease	7	0	+	+	+	++	+	38
Fibroadenoma	1	0	++	+	+	++	+	28
Adenofibroma	1	0	0	+	0	++	±	39
Fibrosing adenomatosis	1	0	+	+	+	++	+	28
Intraductal adenoma	1	0	0	+	++	++	++	33
Ovary:								
Papillary adenocarcinoma	1	+	++	±	++	++	+++	33
Undifferentiated carcinoma	2	+	+	±	++	++	++	38
Krukenberg tumor	4	----------	+	----------	+	----------	++	47
Uterus:								
Leiomyoma	4	0	0	0	0	++	+	40
Prostate:								
Undifferentiated carcinoma	2	0	+	0	0	++	++	63
Benign hypertrophy	15	0	0	0	0	++	++	64
Kidney:								
Grawitz tumor (hypernephroma)	8	++++	++	±	+	++	+	49
Urinary bladder:								
Papillary carcinoma	3	0	0	0	0	++++	++	69
Skin:								
Malignant melanoma	1	0	0	0	0	++	+	33
Epidermoid carcinoma	1	0	0	0	0	++	±	45
Papilloma	1	0	0	0	0	++	+++	30
Fibroma	1	0	0	0	0	++	++	45
Central nervous system:								
Chordoma	1	0	±	0	0	++++	++	72
Protoplasmic astrocystoma	1	0	0	0	0	++++	±	10
Neurofibroma	1	0	0	0	0	++++	+++	40
Unclassified glioma	1	0	0	0	0	++++	++++	45
Lymph nodes:								
Leukemia	3	0	±	+	±	++	+	28
Lymphoma	2	0	0	+	±	++	+	57
Lymphosarcoma	1	0	0	+	±	++	+	72
Large round-cell sarcoma	1	0	0	+	±	++	+	72
Thyroid:								
Papillary carcinoma	1	0	0	0	0	++	++	12
Cyst	1	0	0	0	0	++	+	56
Colloid goiter	1	0	0	0	0	++	±	20
Hyperplasia and involution	5	0	0	0	0	++	++	43
Liver:								
Hepatoma	1	+	0	+	0	+	0	57
Pancreas:								
Adenocarcinoma	2	0	0	0	0	+	±	50
Other tumors:								
Parathyroid adenocarcinoma	1	0	0	0	0	+	±	53
Lung epidermoid carcinoma	1	0	0	0	0	++	+	74
Parotid gland mixed tumor	1	0	0	0	0	++	++	30
Laryngeal polyp	1	0	0	0	0	++	++	34
Capillary hemangioma	1	++	0	0	±	++	+	4
Osteogenic sarcoma	1	++++	++++	+++	+++	++	++	58

[1] The alkaline phosphatase content was estimated by inspection of 4–6 sections of each tumor, fixed and stained under constant conditions and concentrations of reagents.

The symbols which indicate—

0, no stain;
±, rare granular deposit of stain;
+, light granular deposits or faint diffuse stain;
++, moderate stain;
+++, heavy stain; and
++++, intense staining comparable to that seen in renal tubular epithelium, represent an average wherever the results are given for more than one of the same type of tumor.

[2] Estimations of normal phosphatase content were usually made from adjacent areas of normal tissue in the same sections, as well as from numerous normal specimens.

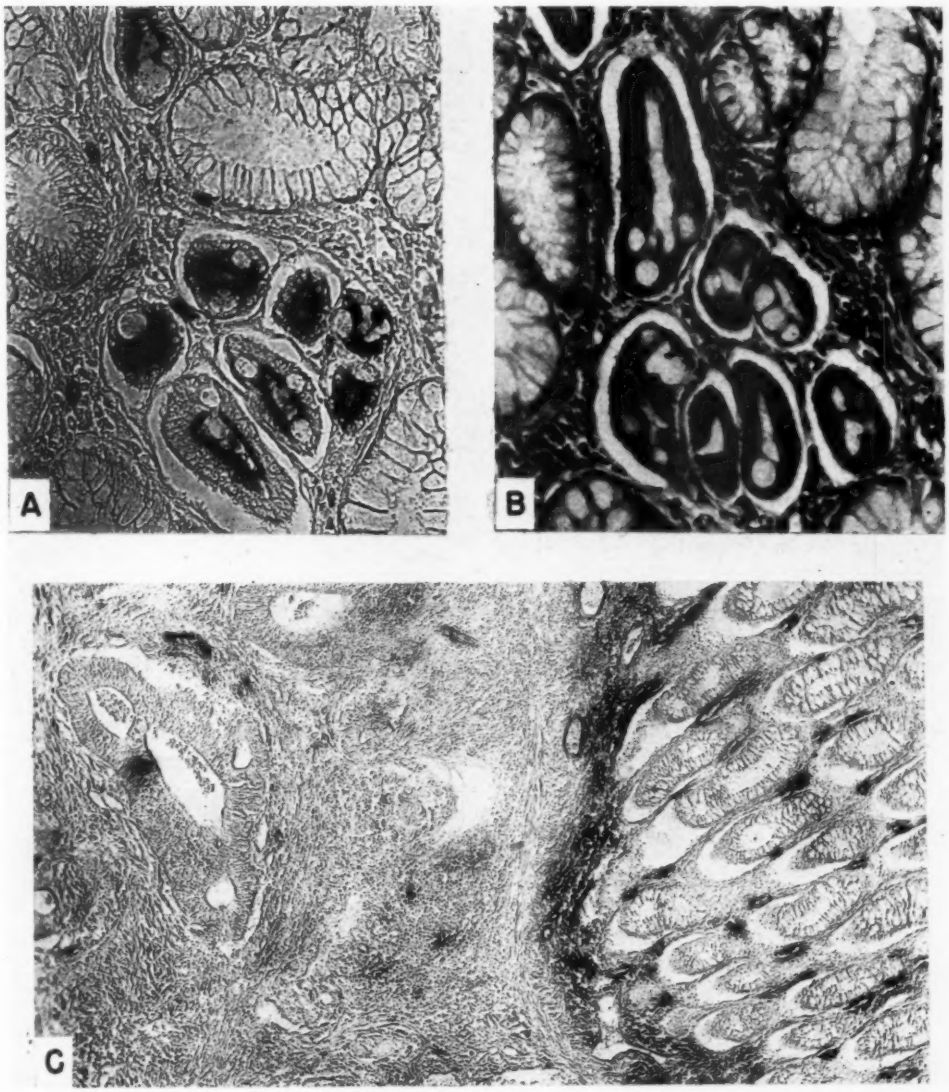

FIGURE 3.—*A*, Human stomach papilloma. Alkaline phosphatase is concentrated in the cuticular border and Golgi region of the cells lining the tumor acini, and in blood vessels. Normal mucus-producing cells of the stomach are free of phosphatase. × 230. *B*, Adjacent section stained with eosin-hematoxylin. The papilloma cells which contained phosphatase are strongly eosinophilic. × 240. (S–46–2480). *C*, Human rectum and rectal adenocarcinoma. The tumor does not contain phosphatase. Few blood vessels in the tumor contain alkaline phosphatase, as compared with the rich network of blood vessels in the normal mucosa and its basal layer (at right). × 110. (S–46–2498).

brane of some cystic glands (fig. 4), and fine granular stain was seen inside many epithelial cells and their nuclei.

In a fibroadenoma, the basement membrane, vascular endothelium, and epithelial cells showed phosphatase activity (fig. 5, *A*). In an adenofibroma, phosphatase activity was absent from the whorls of fibrous tissue, which comprised the bulk of the tumor, but could be found in small amount in the specialized connective tissue in the basement membrane of some of the glands and in the vascular endothelium. In a fibrosing adenomatosis, the alkaline phosphatase was distributed in a pattern similar to that in the fibroadenoma (fig. 5, *B*). An intraductal adenoma (fig. 6) contained phosphatase only in the surrounding stroma and blood vessels. This patient's subsequent course proved the lesion to be carcinoma.

Ovary

Three ovarian tumors appeared to be rich in alkaline phosphatase. A papillary adenocarcinoma showed moderate to profuse phosphatase activity in the cytoplasm of tumor cells, particularly at the cell periphery in tumor cords or at the luminal border of cells in glandular formations. Some staining was also noted in tumor-cell nuclei. Vascular endothelium of this tumor was well-stained.

An undifferentiated carcinoma showed some phosphatase activity in tumor cells and strong activity in the fibrous stroma and vascular endothelium (fig. 7). The 38-year-old patient from whom this tumor was removed received several injections of iodinated polysaccharide (*12*) from *Serratia marcescens*, and a specimen of the tumor examined after injection revealed no change in phosphatase pattern or content (case history 3) (*13*).

Another patient, who had had a gastrectomy for carcinoma 2 years earlier, had oophorectomy for what was considered to be a Krukenberg tumor (fig. 8). Abundant alkaline phosphatase was seen in the tumor cells and fibrous stroma. The irregular pattern and distribution of phosphatase was similar to that of other ovarian tumors (fig. 7) and of normal ovary. In view of the weak or absent phosphatase activity noted in stomach carcinoma, the evidence is strong that this undifferentiated tumor was of ovarian and not of gastrointestinal origin.

Four Krukenberg tumors were examined. The primary lesions were, respectively, adenocarcinoma of the stomach, colloid carcinoma of the descending colon (two), and carcinoma simplex of the breast.

The ovarian metastasis from the adenocarcinoma of the stomach (gastrectomy 11 months earlier) was seen as a well-differentiated tumor. Weak phosphatase activity was noted in occasional tumor cells, but the fibrous stroma between the tumor glands and the blood vessels was rich in alkaline phosphatase (fig. 9, *A*). The high content of phosphatase in the fibrous stroma is consistent with the fact that the stroma was of ovarian origin, since the stroma of gastric carcinoma is free of alkaline phosphatase. The low content of phosphatase in the tumor cells is consistent with their gastric origin, since gastric adenocarcinoma may contain slight amounts of phosphatase whereas ovarian carcinoma is rich in phosphatase.

The ovarian metastases from the two colloid carcinomas of the colon (colon resections 2 and 8 months earlier) exhibited no enzyme in the tumor parenchyma and only faint staining of the stroma and blood vessels. This is in conformity with the phosphatase activity in primary colonic carcinoma (fig. 3, *C*). Similarly, the ovarian metastases from breast carcinoma (radical mastectomy 7 years earlier) showed alkaline phosphatase only in some of the blood vessels.

This type of study should prove useful in determining whether undifferentiated carcinomas found in the ovary are of ovarian or metastatic origin.

Uterus

Four leiomyomas of the uterus were found to have phosphatase only in vascular endothelium and to a degree found in normal uterus. Since endometrium contains phosphatase, tumors of endometrial origin might be expected to have phosphatase. None were included in the present series.

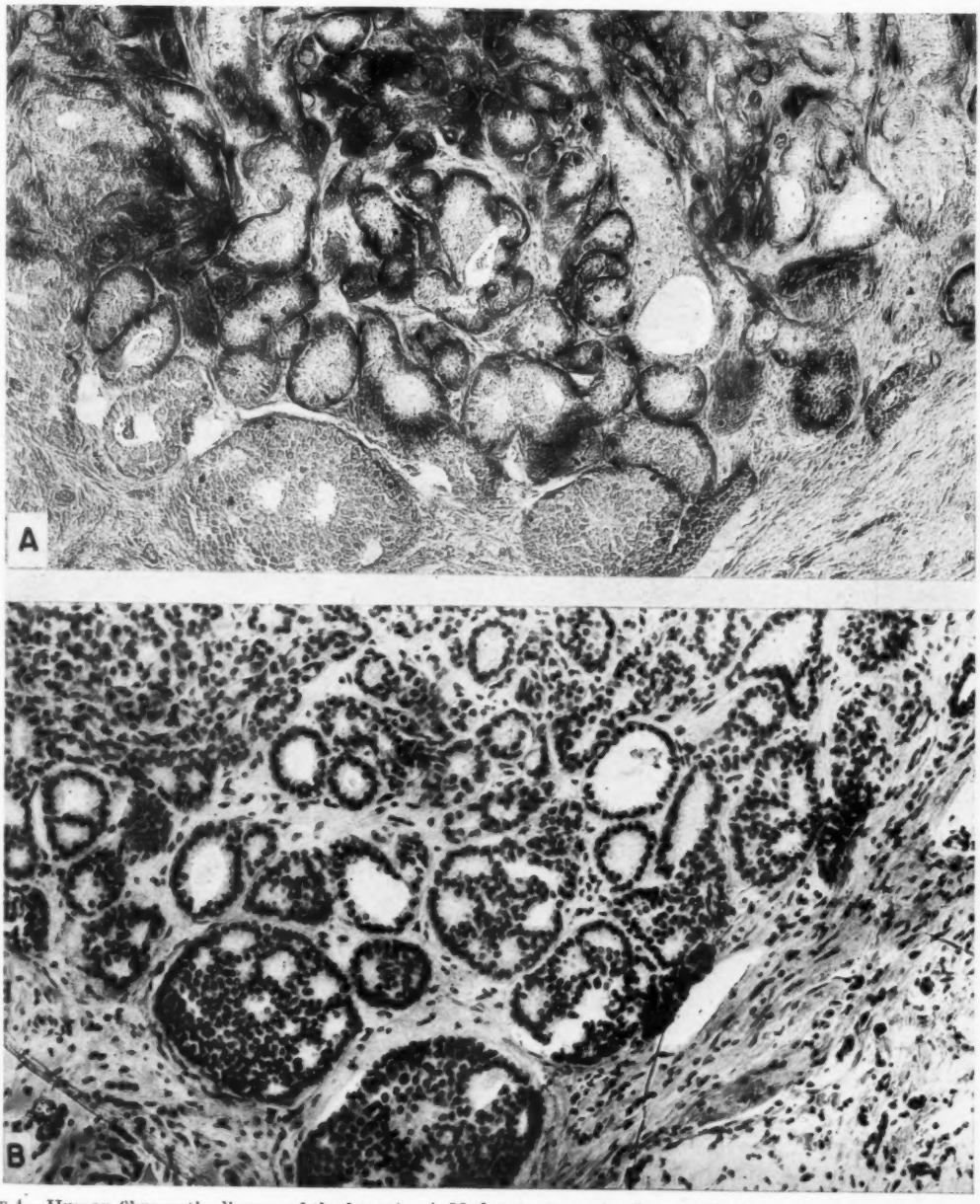

FIGURE 4.—Human fibrocystic disease of the breast. *A*, Moderate concentration of alkaline phosphatase in the basement membrane of many cystic glands is shown. Some of the epithelial cells and nuclei are also stained for phosphatase. Carcinoma unstained for phosphatase is at both lower corners. × 200. *B*, Eosin-hematoxylin stain. × 200. (8-46-2395).

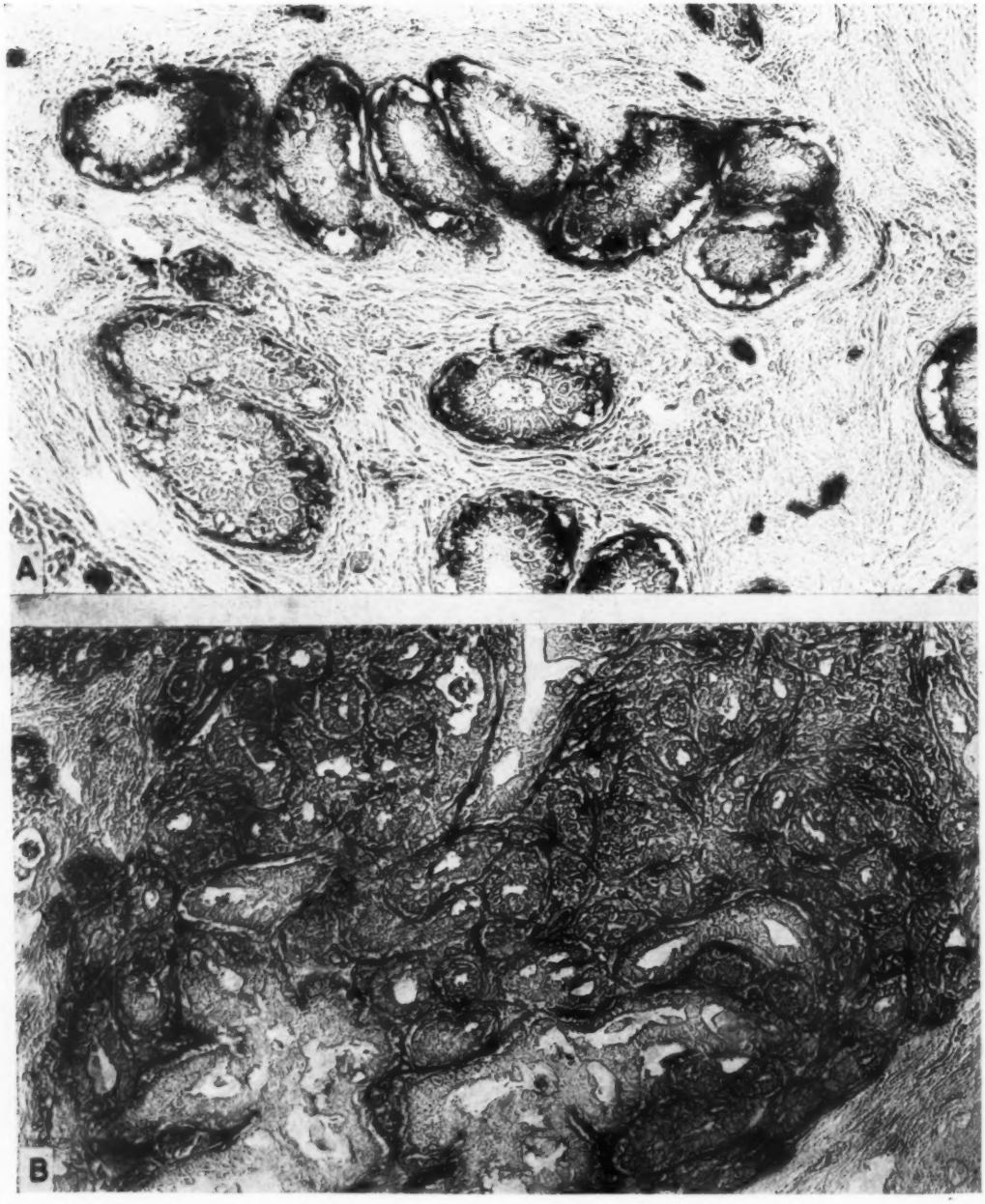

FIGURE 5.—*A*, Human fibroadenoma of the breast. Alkaline phosphatase is abundant in the epithelial cells, basement membrane, and vascular endothelium, as compared with normal breast tissue. × 400. (S–46–2337). *B*, Human fibrosing adenomatosis of the breast. Alkaline phosphatase is particularly abundant in the basement membrane but is also present in the epithelial cells and stroma. × 200. (S–47–932).

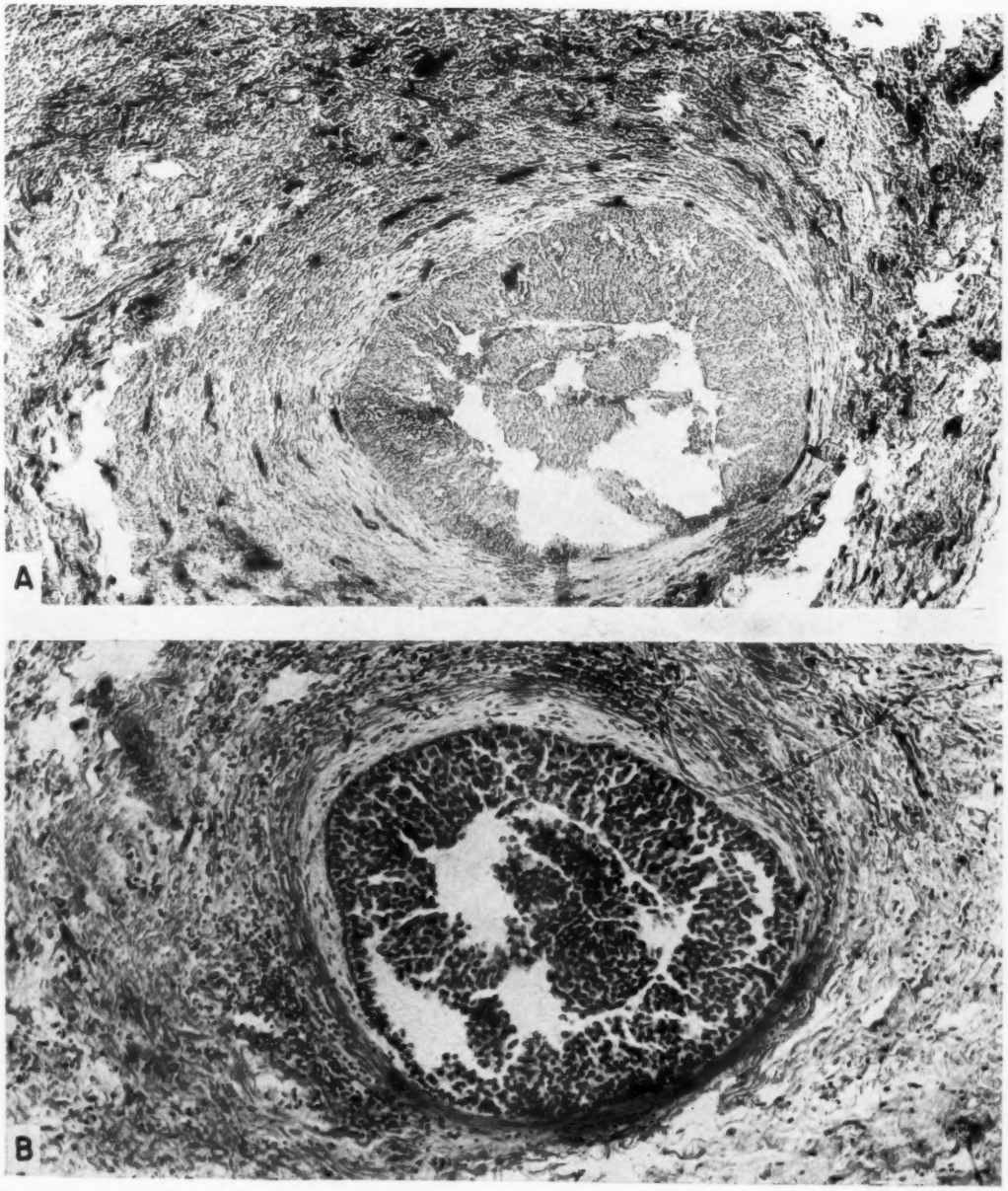

FIGURE 6.—Human intraductal adenoma of the breast. *A*, Alkaline phosphatase is present in the periductal fibrous stroma and in blood vessels. The adenoma cells do not contain phosphatase. × 125. *B*, Eosin-hematoxylin stain. × 125. (S–46–2468).

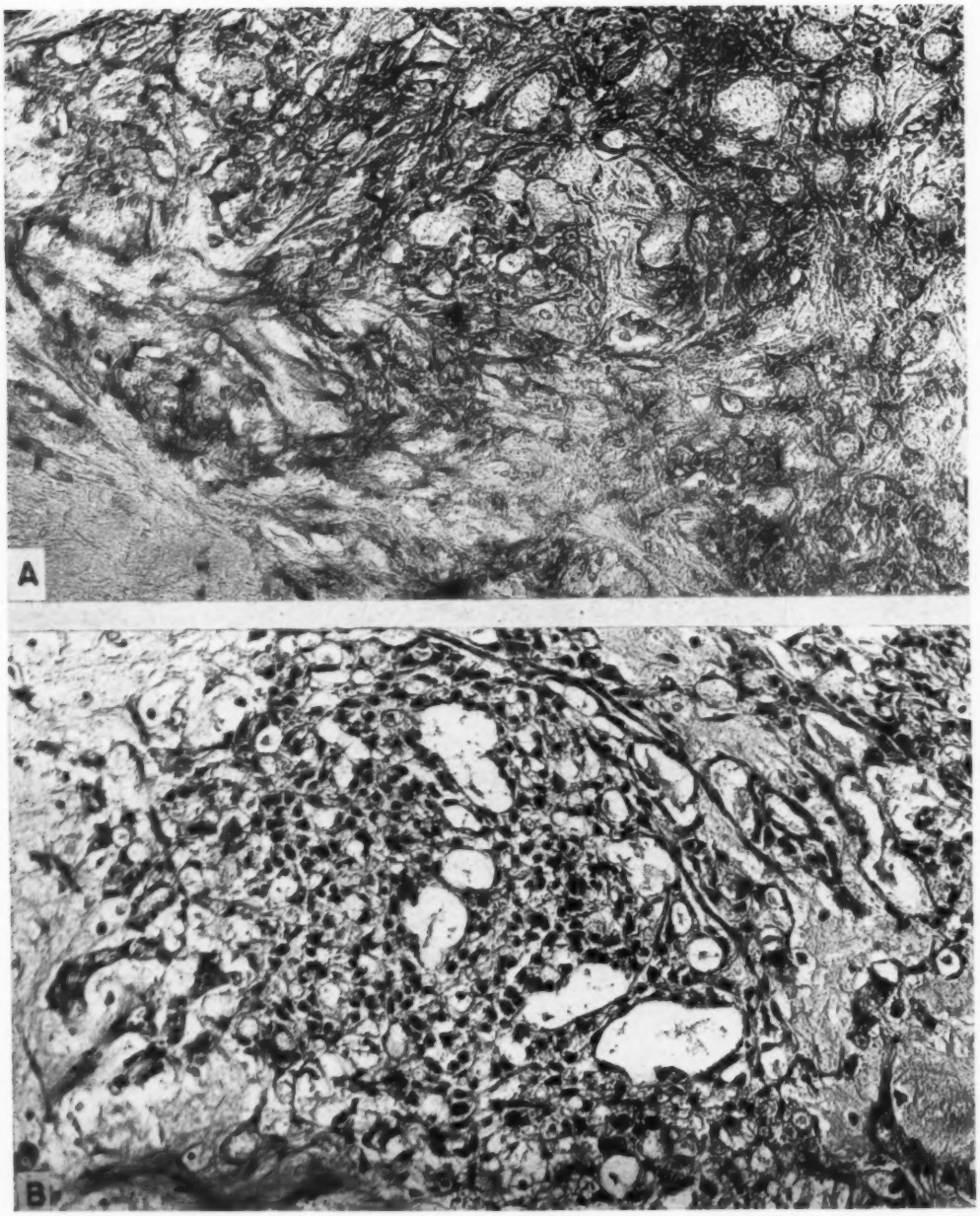

FIGURE 7.—Human undifferentiated carcinoma of the ovary. *A*, Alkaline phosphatase activity is moderate in tumor cells and strong in fibrous stroma and blood vessels. × 200. *B*. Eosin-hematoxylin stain. × 200. (S–47–63.)

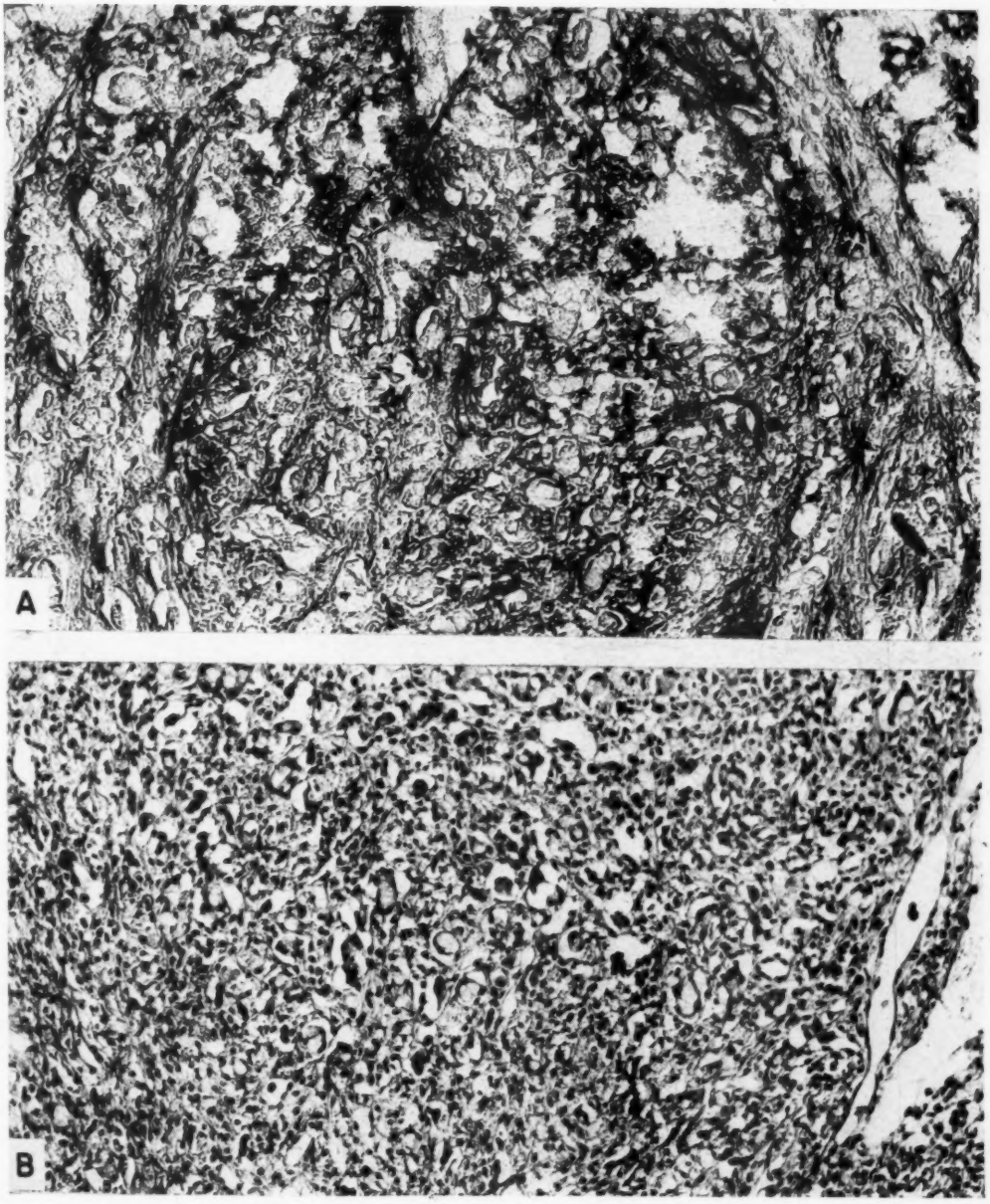

FIGURE 8.—Human undifferentiated carcinoma of the ovary (? Krukenberg tumor from stomach). *A*, Alkaline phosphatase is present not only in the fibrous stroma but also in many tumor cells. The pattern is similar to that of the ovarian carcinoma shown in figure 7. × 200. *B*, Eosin-hematoxylin stain. Some cells suggestive of signet-ring cells (at center) are scattered among the pleomorphic cells. × 200. (S–47–277).

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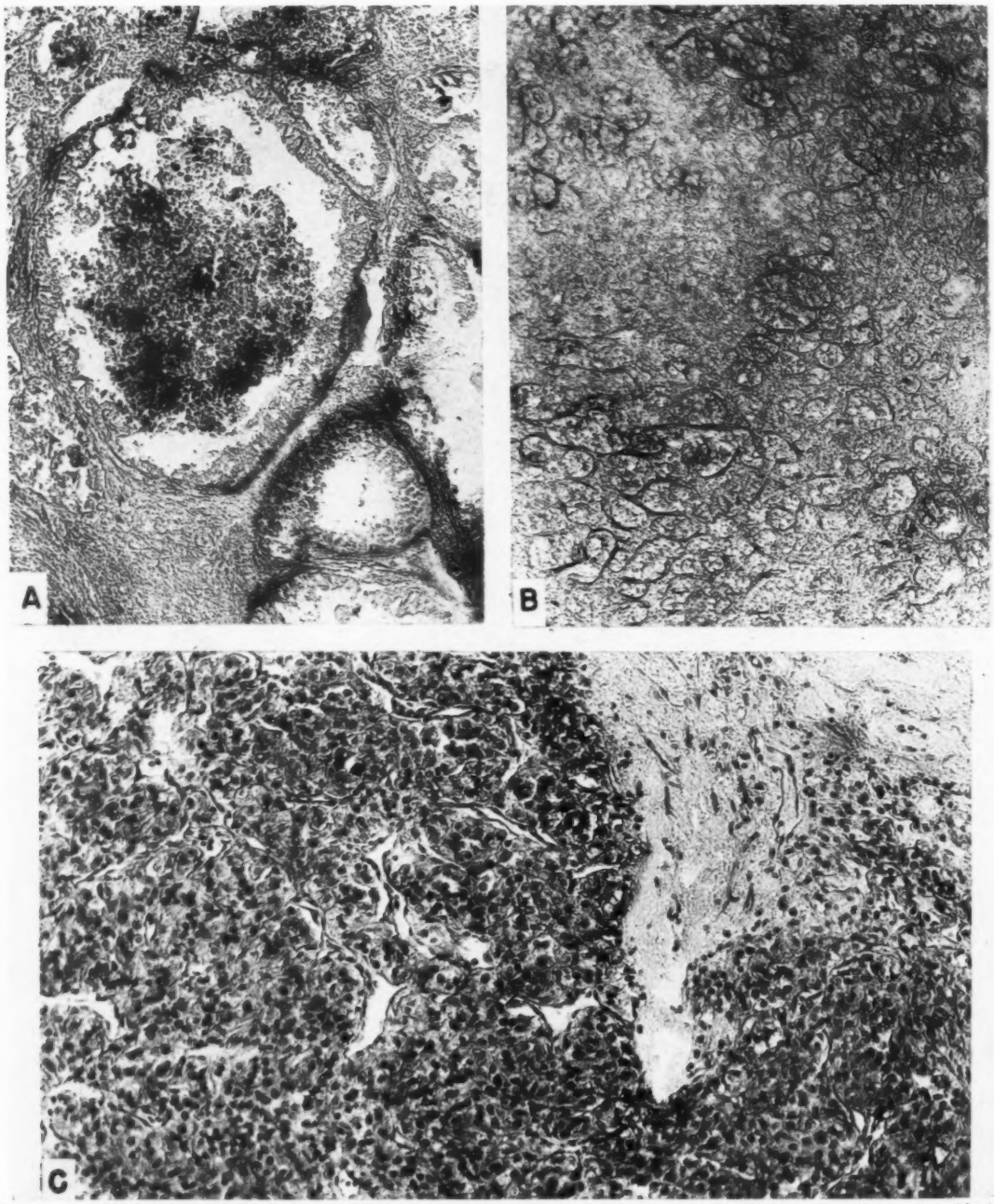

FIGURE 9.—*A*, Human Krukenberg tumor of the ovary (gastric adenocarcinoma). Some alkaline phosphatase is evident in some of the epithelial cells. Otherwise heavy staining is seen only in necrotic tissue, blood vessels, and in the fibrous stroma next to the tumor glands. × 100. (Brigham A–47–76). *B*, Human Grawitz tumor (hypernephroma). Alkaline phosphatase appears as a delicate blush involving cytoplasm and fibrillary stroma in isolated areas of the tumor. × 100. (S–47–1300). *C*, Human renal-cell carcinoma from tumor shown in figure 10. This portion of the tumor is less differentiated and contains less phosphatase than is seen in figure 10 but more phosphatase than is seen in the other Grawitz tumors (fig. 9, *B*). Nuclei stained with hematoxylin. × 200.

Prostate

Only one of two prostatic carcinomas (undifferentiated) exhibited moderate alkaline phosphatase concentration in the cytoplasm and nuclei of a small number of tumor cells. The vascular endothelium had about the same amount of phosphatase as normal prostate. In 15 cases of benign prostatic hypertrophy, alkaline phosphatase was observed only in vascular endothelium.

Kidney

Specimens from eight Grawitz tumors (hypernephroma) were examined. Grossly the tumor tissue presented the characteristic yellow-orange color with mottled areas of hemorrhage and necrosis. Microscopically, seven of these tumors consisted of large cells with clear, foamy cytoplasm, not arranged in definite acinar or cordlike masses, and supported by a fine, fibrillary, interstitial network. The alkaline phosphatase in these tumors was found as finely staining strands in the interstitial matrix and as fine granular deposits inside the cytoplasm of scattered nests of cells (fig. 9, *B*). Except for the fact that these undifferentiated tumors contained alkaline phosphatase, no further similarity to the phosphatase pattern in the kidney could be seen.

The eighth tumor was well-differentiated in many areas into cords and glands. The tumor cells were very rich in alkaline phosphatase (figs. 9, *C*, and 10). The enzyme was concentrated at the luminal borders and supranuclear areas of the tumor cells of well-differentiated areas forming a pattern similar to that seen in the proximal convoluted tubule of the kidney. This is suggestive evidence for the cell of origin of this tumor. The portion of the tumor shown in figure 9, *C*, was less well-differentiated than that shown in figure 10 and contained less alkaline phosphatase. The concentration of phosphatase, however, was greater than that in the other less differentiated Grawitz tumors illustrated in figure 9, *B*.

Urinary Bladder

Three papillary carcinomas of the bladder were devoid of alkaline phosphatase. The intensity of staining of vascular endothelium was variable.

Skin

No alkaline phosphatase was noted in the tumor cells of a malignant melanoma, an epidermoid carcinoma, a papilloma, and a fibroma. With the exception of the epidermoid carcinoma, the enzyme in the vascular endothelium was normal.

Central Nervous System

No phosphatase was seen in a chordoma, protoplasmic astrocytoma, or neurofibroma. Some phosphatase was seen in the fibrillary processes of a glioma (unclassified). Vascular endothelium stained poorly in the protoplasmic astrocytoma.

Lymph Nodes

Phosphatase activity was demonstrated in blood vessels and fibrous stroma. Three leukemic lymph nodes, two nodes containing malignant lymphoma, one lymphosarcomatous metastasis to liver, and one large, round-cell sarcoma contained slightly less enzyme than normal lymph-node tissue. In one leukemic node, occasional small round cells were stained as well as blood vessels and stroma.

Thyroid Gland

Phosphatase activity occurred only in blood vessels in one papillary carcinoma, one cyst, one colloid goiter, and five involution nodules of the thyroid.

Liver

One hepatoma in a cirrhotic liver was free of phosphatase. The portal areas of the cirrhotic liver were particularly rich in phosphatase.

Pancreas

Two adenocarcinomas revealed slight phosphatase activity in blood vessels.

Other Tumors

The following tumors failed to show alkaline phosphatase in the neoplastic cells: one adenocarcinoma of the parathyroid, one epidermoid carcinoma of the lung, one mixed tumor of the parotid gland, one laryngeal polyp, and one capillary hemangioma. Vascular endothelium stained weakly in the parathyroid tumor and moderately heavily in the other tumors, as compared with the

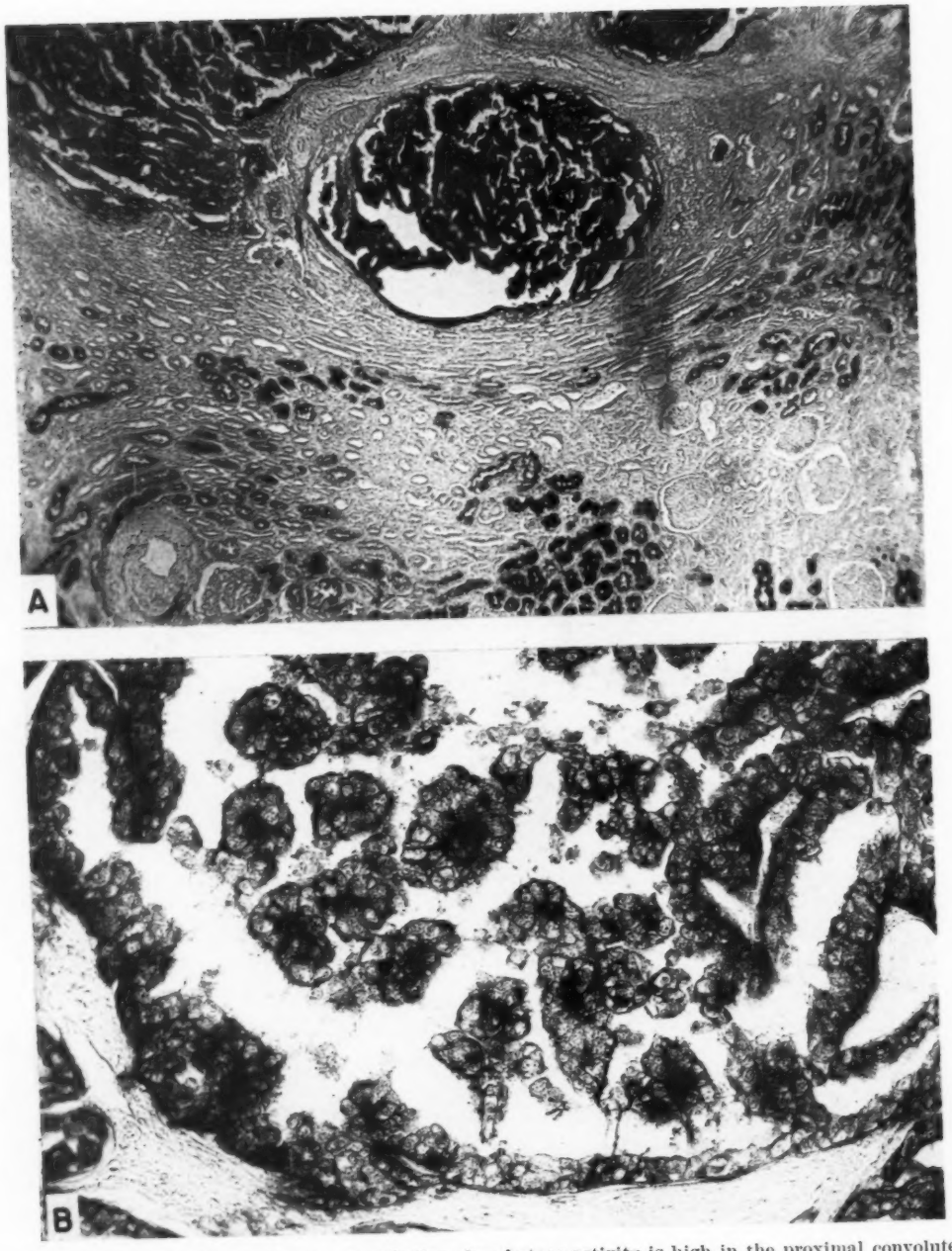

FIGURE 10.—Human renal-cell carcinoma. *A*, Alkaline phosphatase activity is high in the proximal convoluted tubules of the kidney and in the tumor cells. Glomeruli are free of phosphatase. × 54. *B*, Higher power; phosphatase distribution in the acini of the tumor is similar to that of kidney tubular epithelium. × 260. (S–46–2341.)

tissues of origin. An osteogenic sarcoma of the femur was rich in alkaline phosphatase throughout.

Mouse Tumors

Five tumors [10] of spontaneous origin in C57 black mice and maintained by transplantation in this strain of mouse were stained for alkaline phosphatase.

A fibrosarcoma (L946AII) of bone origin but not osteogenic failed to reveal any alkaline phosphatase activity. An osteogenic sarcoma (C1977) showed marked activity. The tumor cells were heavily stained, the nuclei more than the cytoplasm. Cells of the stroma stained darkly, particularly along the course of the fibrils. A myxoliposarcoma (C1853) showed diffuse and faint staining of all the cellular components of the tumor, more marked in the large, round cells than in the spindle-shaped ones. The nuclei of both cell types were characterized by heavy granular staining of the chromatin network and nucleoli. Vascular endothelium did not stain. An adenocarcinoma of breast (E0771) showed moderate staining of epithelial cells and less of fibrous stroma. Dye was concentrated in supranuclear regions of the epithelial tumor cells and at luminal borders of acini. The nuclei contained dark, granular deposits of dye. Vascular staining was slight. A myeloid leukemia (C1498) obtained as a nodule gave evidence of activity only in the blood vessels.

DISCUSSION

The techniques of Gomori (*6*) and Takamatsu (*7*) for demonstrating alkaline phosphatase involve enzymatic hydrolysis of phosphoric esters of glycerol, glucose, or other naturally occurring substances in the presence of calcium ions, with the consequent precipitation of calcium phosphate. These insoluble deposits are subsequently made visible either by Von Kossa's method (*14*) of transforming calcium phosphate to silver phosphate, the latter subsequently turning brown on exposure to sunlight, or else by changing calcium phosphate via cobalt phosphate to black cobalt sulfide. Although the method is laborious, the use of naturally occurring phosphates as substrates makes the method of particular value in comparing phosphatase activity on various types of phosphoric esters. Advantages which may be pointed out for the method of Menten et al. (*1*), as here simplified, are as follows:

(1) The shorter period of incubation required for developing good color (15 min. compared with hours) indicates that fewer substrate molecules need be hydrolyzed to give good visible effects. Therefore, the method has greater sensitivity.

(2) The absence of naphthol in tissue makes control slides unnecessary, while methods based on pigments formed from phosphates require control slides.

(3) The greater insolubility in water of the azo dyes formed in the tissue, as compared with formed calcium phosphate (which is soluble in 100 cc. of cold water to the extent of 2.5 mg.) makes for sharp pictures and less danger of migration of the pigments within the tissues. Further danger of artefacts due to migration are introduced in conversion of calcium phosphate to silver or cobalt phosphate. The possibility of special tissue affinities for these heavy metals increases the danger of artefacts.

(4) The development of pigment can be watched and stopped when optimal.

(5) Bone can be readily decalcified after staining for phosphatase. Although the stained block cannot be embedded in paraffin due to lipoid solubility of the azo dye, frozen sections can be made (fig. 1, *D*).

In general the results obtained by the azo dye technique correspond closely to those reported in the literature with the older method.

The deposits of azo dye at sites of alkaline phosphatase activity appear in two forms. Small granules of the dye appear within the first 60 seconds of incubation. At the end of 2 minutes a diffuse stain appears in the area of dark granules. This diffuse stain becomes progressively deeper, reaching a maximum 20 minutes later. The depth of the stain varies, under constant conditions presumably depending on the enzyme concentration. The stain is not always confined to cellular boundaries but is present in the intercellular matrix of many tissues as a faint haze. This is a reflection of the presence of minute quantities of alkaline phosphatase in the intercellular fluids and ground substance. That this is not due to diffusion of dye

[10] Obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine. The identification numbers are given after each tumor.

itself, once it is formed, as indicated by the observation that the process does not continue after removal of the section from the substrate or during the incubation in the mounting fluid of the permanent sections during the year that we have observed them. Although alkaline phosphatase has not been reported in interstitial fluid, it may be expected to occur in areas close to phosphatase rich cells, since it is present in blood serum. Postmortem diffusion of the enzyme itself would be expected to operate less extensively during the short period of incubation in aqueous medium required by this method than with longer periods of incubation (hours) required by Gomori's method.

The observation that the blood vessels in tumor tissue are relatively devoid of alkaline phosphatase has been reported only for neoplasms of the central nervous system (*10*). With the exceptions given in table 1, this appeared to be a general though not diagnostic phenomenon. Young blood vessels of granulation tissue are characterized by a diffuse even though quantitatively light staining of their endothelium in contrast with the absence of phosphatase in the blood vessels of most cancer tissue. It seems unlikely, therefore, that the relative absence of alkaline phosphatase in vascular endothelium of tumor tissue is due to young, newly formed blood vessels. Experimental studies (*15*) of vascularization of transplanted tumors indicate that the transplant has the capacity of eliciting from the host continuous development of new capillary endothelium. By ordinary histological methods young vessels in malignant tissue cannot be differentiated from those in normal neighboring tissue. This is also the case in human tumors. Yet alkaline phosphatase determinations suggest physiological differences.[11]

Similarly, alkaline phosphatase content of kidney tissue is a sensitive index of its functional state (*18*), and in experimental hydronephrosis disappearance of phosphatase from kidney tubules is not accompanied by discernible histologic changes (*19*).

The presence of alkaline phosphatase in normal breast tissue, in fibroadenoma, and in the basal layers of the glands in some forms of fibrocystic disease of the breast in contrast with the absence of phosphatase in all forms of breast carcinoma may prove to be of diagnostic aid, especially when rapid diagnosis is required from frozen sections.

The presence of alkaline phosphatase in ovarian carcinomas in contrast with its absence from carcinomas of the stomach, colon, and rectum should prove helpful in determining whether the tissue of orgin of a Krukenberg tumor is ovary or not. The carcinomas of gastrointestinal origin growing in ovary, examined in the present series, were, with rare exceptions, devoid of phosphatase in the tumor cells, although traces of phosphatase were seen in the stroma (probably of ovarian origin).

The pattern of alkaline phosphatase in a renal cell carcinoma was so strikingly similar to that of the proximal convoluted tubular epithelium of the normal kidney as to suggest that the tumor was of tubular cell origin (fig. 10). A less well differentiated area of this tumor and all of the other Grawitz tumors contained phosphatase, but the pattern of distribution did not strikingly resemble that in the tubular epithelium of the kidney. Evidence concerning the cell of origin of Grawitz tumors may be provided by further studies of this type. Greenstein's work (*20*) indicates that although neoplasms usually have less, or rarely more, enzymes than the tissue of origin, enzymes which are not present in the tissue of origin, do not appear in neoplastic tissue.

SUMMARY

The method of Menten, Junge, and Green for the histochemical demonstration of alkaline phosphatase has been simplified and made practicable. The method is compared with the widely used methods of Gomori and Takamatsu. The distribution of alkaline phosphatase in normal tissues is discussed, and its distribution in 129 human tumors is given.

On the basis of a limited number of tumors of each type studied, it may be tentatively concluded that (1) the blood vessels of neoplastic tumors have less alkaline phosphatase than the blood vessels of normal tissue; (2) carcinoma cells of kidney and ovary and sarcomas of bone contain alkaline phosphatase; (3) breast carcinomas are

[11] These findings may be related to the observations of destructive effects by a tumor necrotizing polysaccharide of bacterial origin upon the blood vessels of sarcomas of mice (*12, 16*) and man (*17*), while leaving the blood vessels of normal tissues relatively unaffected.

devoid of alkaline phosphatase in contrast with various types of benign breast tumors; and (4) carcinoma cells of the gastrointestinal tract are, with rare exceptions, devoid of alkaline phosphatase in contrast with carcinoma cells of the ovary.

Krukenberg tumors so undifferentiated as to defy classification by ordinary histological examination may be proved to be of ovarian origin on the basis of their alkaline phosphatase content and pattern.

REFERENCES

(1) MENTEN, M. L., JUNGE, J., and GREEN, M. H.: A coupling histochemical azo dye test for alkaline phosphatase in the kidney. J. Biol. Chem. 153: 471–477 (1944).

(2) SAUNDERS, K. H.: The aromatic diazo-compounds and their technical applications. Longmans, Green & Co., New York (1936).

(3) STAFFORD, R. O., and ATKINSON, W. B.: Effect of acetone and alcohol fixation and paraffin embedding on activity of acid and alkaline phosphatases in rat tissues. Science 107: 279–281 (1948).

(4) MICHAELIS, L.: Diethylbarbiturate buffer. J. Biol. Chem. 87: 33–35 (1930).

(5) MALLORY, F. B.: Pathological technique. W. B. Saunders, Philadelphia (1938).

(6) GOMORI, G.: Microtechnical demonstration of phosphatase in tissue sections. Proc. Soc. Exper. Biol. & Med. 42: 23–26 (1939).

(7) TAKAMATSU, H.: Histologische und biochemische Studien über die Phosphatase: I. Histochemische Untersuchungsmethodik der Phosphatase und deren Verteilung in verschiedenen Organen und Geweben. Tr. Jap. Path. Soc. 29: 492–498 (1939).

(8) KABAT, E. A., and FURTH, J.: A histochemical study of the distribution of alkaline phosphatase in various normal and neoplastic tissues. Am. J. Path. 17: 303–318 (1941).

(9) WACHSTEIN, M., and ZAK, F. G.: Distribution of alkaline phosphatase in the human liver. Arch. Path. 42: 501–516 (1946).

(10) LANDOW, H., KABAT, E. A., and NEWMAN, W.: Distribution of alkaline phosphatase in normal and in neoplastic tissues of the nervous system. Arch. Neurol. & Psychiat. 48: 518–530 (1942).

(11) DEANE, H. W., and DEMPSEY, E. W.: The localization of phosphatases in the Golgi region of intestinal and other epithelial cells. Anat. Rec. 93: 401–417 (1945).

(12) SELIGMAN, A. M., SHEAR, M. J., LEITER, J., and SWEET, B.: Chemical alteration of polysaccharide from *Serratia marcescens*. I. Tumor-necrotizing polysaccharide tagged with radioactive iodine. J. Nat. Cancer Inst. 9: 13–18 (1948).

(13) SACK, T., and SELIGMAN, A. M.: Chemical alteration of polysaccharide from *Serratia marcescens*. II. Effect of iodopolysaccharide in patients with malignant tumors. J. Nat. Cancer Inst. 9: 19–34 (1948).

(14) KOSSA, J. v.: Ueber die im Organismus künstlich erzengbaren Verkalkungen. Beitr. z. path. Anat. u. z. allg. Path. 29: 163–202 (1901).

(15) ALGIRE, G. H., and CHALKLEY, H. W.: Vascular reactions of normal and malignant tissues in vivo. I. Vascular reactions of mice to wounds and to normal and neoplastic transplants. J. Nat. Cancer Inst. 6: 73–85 (1945).

(16) SHEAR, M. J.: Chemical treatment of tumors. IX. Reactions of mice with primary subcutaneous tumors to injections of a hemorrhage-producing bacterial polysaccharide. J. Nat. Cancer Inst. 4: 461–476 (1944).

(17) HOLLOMAN, H. L.: Reactions of patients and of tissues to injection of S. marcescens polysaccharide in eight cases of malignant disease. *In* Approaches to Tumor Chemotherapy, pp. 236–284. The Science Press Printing Co., Lancaster, Pa. (1947).

(18) KAY, H. D.: The phosphatases of mammalian tissues. Biochem. J. 22: 855–866 (1928).

(19) WILMER, H. A.: The disappearance of phosphatase from the hydronephrotic kidney. J. Exper. Med. 78: 225–231 (1943).

(20) GREENSTEIN, J. P.: Enzymes in normal and neoplastic animal tissues. *In* Research Conference on Cancer, pp. 192–215. A. A. A. Sc., Washington, D. C. (1945).

MORPHOLOGIC CHANGES IN THE ORGANS OF FEMALE C3H MICE AFTER LONG-TERM INGESTION OF THIOUREA AND THIOURACIL[1,2,3]

By A. J. DALTON, HAROLD P. MORRIS, and CELIA S. DUBNIK, *National Cancer Institute, National Institutes of Health, Public Health Service*

INTRODUCTION

Previous reports concerning the effects of various goitrogenic substances, including thiourea and thiouracil, dealt primarily with changes in the thyroid (*1*) and in the basal metabolic rate (*2*). A few described changes in other organs (*3*), and Baumann and Marine described in detail adrenal (*4*) and vascular (*5*) changes. All these investigators dealt with relatively short term experiments. Our study on the effect of long-continued feeding of thiourea on the mammary glands and mammary-tumor incidence of adult mice (*6*) provided material which showed histologic and cytologic changes in other organs as well as in the thyroid. Some of the more striking effects were reported in a previous article (*7*). The present paper is a more detailed account of these changes and includes also the changes induced with thiouracil ingestion by younger mice continued for still longer periods, reported briefly elsewhere (*8*, *9*).

MATERIALS AND METHODS[3]

THIOUREA EXPERIMENT

Twenty-one 11-month-old C3H female mice were placed on a diet containing 0.25 percent thiourea, which after 13 weeks was increased to 0.375 percent. Twenty-three female C3H mice were placed on the same basic diet without thiourea. Both control and experimental animals were killed when tumors appeared. Thus experimental animals ingested thiourea for periods varying from 3 to 63 weeks, the intervals being 5, 7, 8, 11, 13, 17, 20, 24, 25, 30, and 42 weeks. Thyroid, kidney, uterus, ovary, liver, spleen, heart, thymus, and pancreas were fixed in Zenker-formol, embedded in paraffin, cut at 6μ, and stained with hematoxylin and eosin. Organs of mice that died overnight were preserved in 4 percent formaldehyde. The adrenals, pituitary, and occasionally the thyroid and liver were fixed in Champy's fluid (1 part 2 percent osmic acid, 1 part 1 percent chromic acid, and 1 part 3 percent potassium dichromate) for 24 hours, washed in running tap water for 24 hours, then placed in 2 percent osmic acid at 40° C. for 5 days, embedded in paraffin, and cut at 4μ. In addition, liver and occasionally thyroid were fixed in Regaud's fluid at 3°–5° for 3 days, then placed in 5 percent potassium dichromate for 3 days at 40°, washed, embedded in paraffin, sectioned at 3μ, and stained by Heidenhain's long iron-hematoxylin method. Tissues were stained for iron by a modification of the prussian-blue reaction (*10*).

THIOURACIL EXPERIMENT

One hundred and forty-three virgin female C3H mice were divided into two approximately equal groups, as described (*9*). Their ages varied from 9½ to 10½ weeks and averaged 10 weeks. Both groups of mice received the same adequate basal diet as did the thiourea-fed group. To the diet of one group, 0.375 percent thiouracil was added, which was later increased to 0.5 percent. Some control and experimental animals were killed at selected intervals, while other animals were killed when they appeared to be ill or when tumors appeared.

RESULTS

THYROID

THIOUREA

Changes in the thyroid after ingestion of thiourea were similar to those previously described for other species (*3*). Thyroids from animals on thiourea for short periods (3 weeks) contained follicles lined by tall columnar cells These follicles showed less acidophilic colloid than is found in normal follicles. After longer periods of thiourea ingestion, many follicles were completely collapsed, while others contained small amounts of palely staining colloid. All thyroids of animals receiving thiourea for 5 or more weeks were found to be hyperemic on visual examination, and the majority showed histologic evidence of

[1] Received for publication October 7, 1947.

[2] Presented in part before the Society for Experimental Biology and Medicine, Washington, D. C., February 7, 1946.

[3] The thiouracil in these experiments was supplied by Lederle Laboratories, Pearl River, N. Y., through the courtesy of Dr. S. M. Harding.

[4] For details of diet and treatment, see Morris, Dubnik, and Dalton (*6*).

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intrafollicular hemorrhage. As described previously (7), this condition was followed by the accumulation of iron-positive pigment in the cells lining the follicles. Cells tentatively identified as "parafollicular" cells were first noted in the thyroid of a mouse receiving thiourea for 11 weeks. These cells were extremely large, containing one or more large vacuoles filled with pale-staining, colloidlike material. Occasionally they formed part of a follicle wall, but more frequently they appeared to be parafollicular in position. Their nuclei were frequently pyknotic and eccentrically located. Of 12 animals ingesting the compound for 11 or more weeks, 8 showed numerous "parafollicular" cells in their thyroids.

THIOURACIL

The thyroids of 7 control mice in the thiouracil group were dissected from the larynx and trachea and weighed (table 1). The average weight was 4.5 mg. The weights of the thyroid plus the larynx and the first tracheal ring in 1 group of 7 mice averaged 14.9 mg., and in a second group of 6 animals the larynx and tracheal ring alone averaged 10.0 mg. Thyroid weight in animals receiving thiouracil averaged 10 times that of control mice.

Table 1 and figure 1 show the wide variation in thyroid-gland weight of the thiouracil-treated mice (from 12.3 to 141.5 mg.). Figure 1 also shows that while in general gland weight was proportional to the length of treatment, there was considerable individual variation, particularly in the group treated for more than 300 days.

TABLE 1.—*Organ weights of control and experimental animals*

CONTROL DIET 77

Organ	Number of animals	Average weight	Range
		Milligrams	*Milligrams*
Thyroid	7	4.5	2.6– 7.0
Thyroid and trachea	9	14.9	11.2– 22.5
Ovary	10	7.4	3.2– 12.5
Ovaries and uterus	26	116.5	30.2–242.0
Adrenals	27	7.2	3.9– 12.2
EXPERIMENTAL DIET 145			
Thyroid	55	59.3	12.3–141.5
Ovary	9	4.7	2.0– 7.3
Ovaries and uterus	38	36.3	13.3–110.0
Adrenals	38	4.1	2.1– 6.0

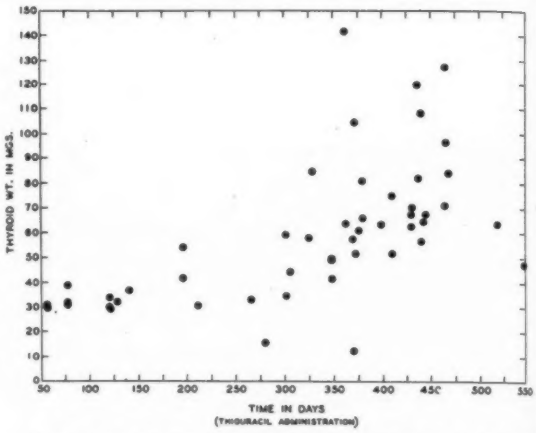


FIGURE 1.—Scatter diagram showing variation of thyroid weight in relation to length of treatment with thiouracil.

During the early period of thiouracil feeding (1 to 6 months), changes in the thyroid gland were very similar to those seen after thiourea ingestion. The colloid lost its acidophilic staining reaction and decreased in amount, first in the center of the gland and later at the periphery. The cells increased in size and acquired a definitely columnar form. The nuclei became larger and relatively vesicular and hence pale blue in hematoxylin-eosin preparations. There appeared to be a decrease in the amount of chromatin in the nuclei. The collapse of the follicles consequent to the disappearance of colloid resulted in a distinct change in the structure of the gland. Under lower power, the change appeared superficially to range from an alveolar to a tubular type of gland (fig. 2, *A* and *B*). In spite of extreme hypertrophy of the gland involving both cellular hypertrophy and hyperplasia, resulting, in some instances, in increases to as much as 25 times the normal weight, no histologic evidence of neoplasia was found. In one case, part of one lobe had broken through the gland capsule, and in another, thyroid tissue was found between the trachealis muscle and the tracheal epithelium. Both of these conditions appeared to have resulted from pressure effects, and in neither case was there evidence of true invasiveness.

With continued thiouracil ingestion, the thyroid retained this hyperplastic state for approximately 12 months, but at about this time a few follicles were found to contain a moderate amount of

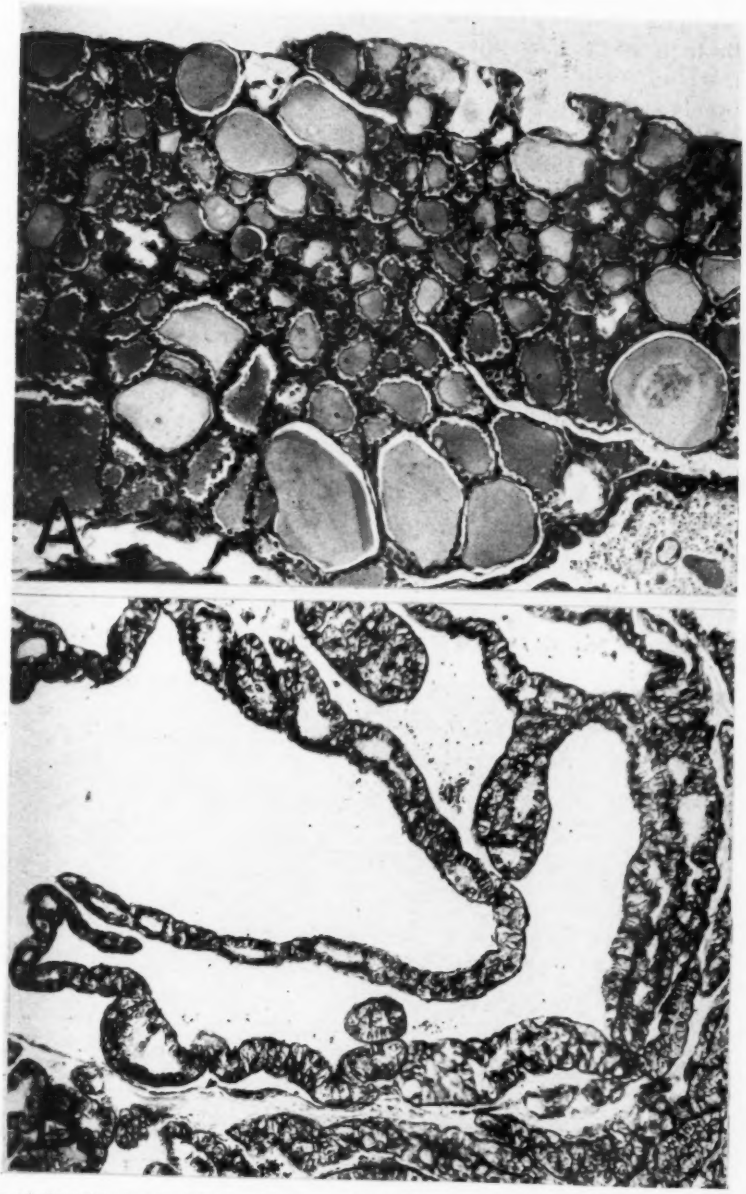


FIGURE 2.—*A*, Section of thyroid gland of control mouse. Zenker-formol fixation and hematoxylin-eosin stain. × 135. *B*, Thyroid of mouse treated with thiouracil for 432 days, showing the gland to be in the form of cords containing flattened follicles. Champy fixation, 5 days post-osmication. × 135.

acidophilic colloid. Later, follicles containing colloid became more numerous. The nuclei of cells forming the walls of these follicles were smaller and more basophilic than normal, and they appeared to contain more chromatin (fig. 7, *A*).

"Parafollicular" or "pale" cells were present in the youngest animals studied (56 days of thiouracil ingestion), reached maximum at about 12 months, and retained this high level for the remainder of the period of ingestion. Figure 3 shows the "pale" cells as they appeared in this later period of treatment.

Multinucleate masses were first found in a thyroid after 119 days' treatment and rather consistently after this (of 43 thyroids from animals treated 119 days or longer, 38 contained these masses). They were found in close association with the follicle walls. The nuclei stained heavily with hematoxylin and were somewhat smaller than the nuclei of normal follicular cells. The cytoplasm surrounding these nuclei was pale-staining and possessed no clear boundary (fig. 4).

Hemorrhage into follicles was found rather frequently in glands from animals treated for less than a year (fig. 5), and this was correlated with the presence of iron-positive pigment in follicular cells during this interval (fig. 6). In animals treated for more than 1 year, pigmented follicular cells were noted in the thyroids of a few, but in none was there evidence of hemorrhage.

Lungs

During the early stages of the experiment with thiouracil, lungs were not examined histologically. However, an animal which had ingested thiouracil for 362 days exhibited numerous, superficial, pearly gray nodules. The lungs of this animal and of the remaining 23 experimental and 9 control mice were fixed for later histologic examination. Ten of the twenty-three experimental mice exhibited similar nodules. All the nodules were identified as thyroid tissue. The period of thiouracil ingestion by these 10 animals varied from 362 to 464 days. Four animals which had been fed thiouracil for longer periods, up to 549 days, did not exhibit nodules. These data are the result of careful examination of a single section containing samples of all lobes of the lung of each animal. Serial sections were not taken.

FIGURE 3.—Thyroid from mouse treated with thiouracil for 348 days, showing numerous "parafollicular" or "pale" cells. Zenker-formol fixation; hematoxylin-eosin stain. × 355.

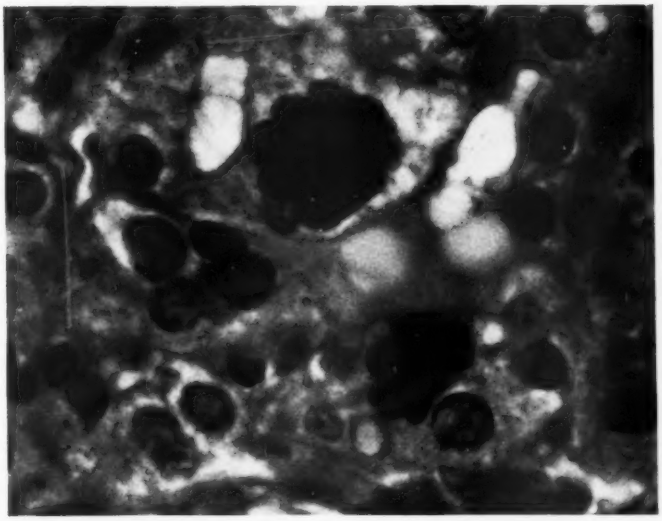


FIGURE 4.—Thyroid from mouse treated with thiouracil for 431 days, showing two multinucleate cells with hyperchromatic nuclei. Zenker-formol fixation; hematoxylin-eosin stain. × 1300.

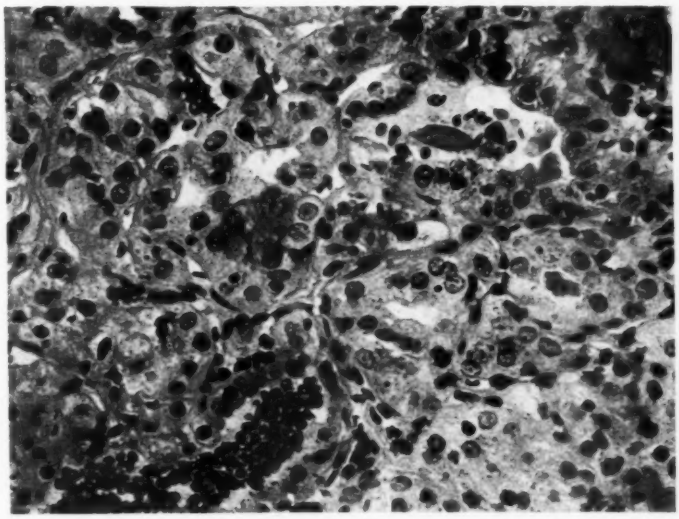


FIGURE 5.—Thyroid from a mouse treated with thiouracil for 119 days, showing old and new hemorrhage in follicles. Zenker-formol fixation; hematoxylin-eosin stain. × 400.

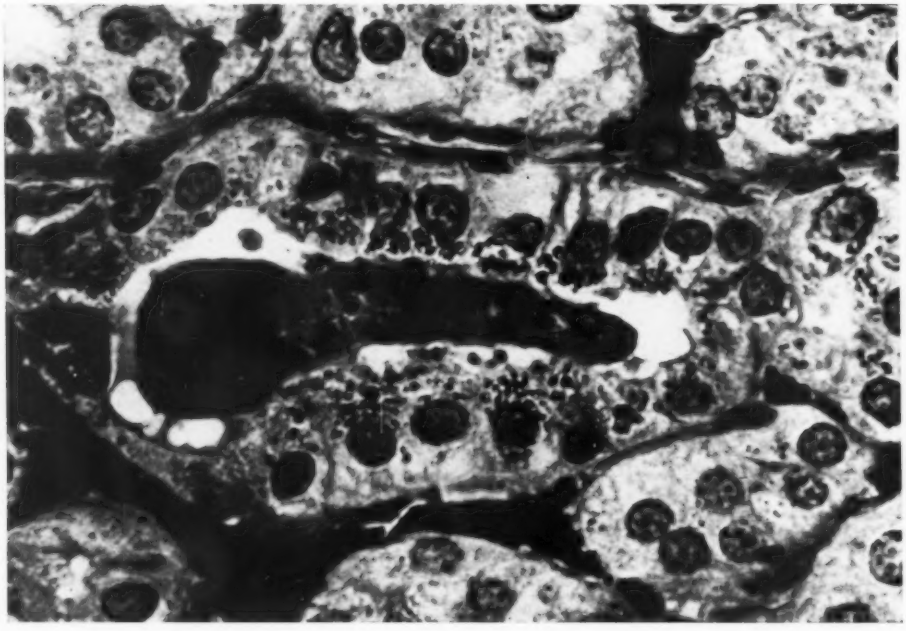


FIGURE 6.—Thyroid from a mouse treated with thiouracil for 119 days, showing large amounts of iron-positive pigment in the distal parts of the follicular cells. Zenker-formal fixation; hematoxylin-eosin stain. × 1040.

The thyroid tissue in these nodules was remarkably similar in histologic appearance to that of the host thyroid. In many cases, the nodules were obviously contained within blood vessels, being surrounded with endothelium. When the host thyroid follicles contained little or no colloid, the follicles in the lung nodules contained little or none. When individual follicles of the host thyroid contained colloid, individual follicles of the lung nodules did also (fig. 7, *A* and *B*).

An unusual type of pigment was in the cells of the respiratory epithelium of the lungs of the majority of the experimental mice examined for it. It was yellowish to brown, with a slight greenish tinge, and negative for iron. It was deposited in the form of relatively large oval to cigar-shaped granules, usually in a position distal to the nucleus (fig. 8). Fourteen of the twenty-three lungs examined contained varying amounts of this pigment.

ADRENALS

The adrenals of a mouse killed after 3 weeks of thiourea ingestion showed distinct alterations. The cortex was somewhat thinner than in the control animals, and many cells in individual columns of the fasciculata showed only minute osmiophilic droplets (fig. 9, *A* and *B*). This change gave a streaked appearance to the fasciculata when viewed under the low power of the microscope. In addition, there was an increase in the number of degenerating cells at the cortico-medullary border. At this stage, the capsule was normal in appearance, being thin and containing few cells.

After ingestion of thiourea for longer periods, e. g., 17 weeks, the whole fasciculata rather than individual cords was found to be altered. At this stage, the fasciculata cells contained only minute, dustlike particles of osmiophilic material, in contrast with the usually large droplets in normal, untreated tissue. On the other hand, the zona glomerulosa appeared to contain more than the normal amount of osmiophilic material. This apparent increase was emphasized by the sharp reduction in lipid at the border of the fasciculata. The capsule was thicker and contained more cells than that of the control gland. Many of the inner capsule cells contained a few small osmiophilic

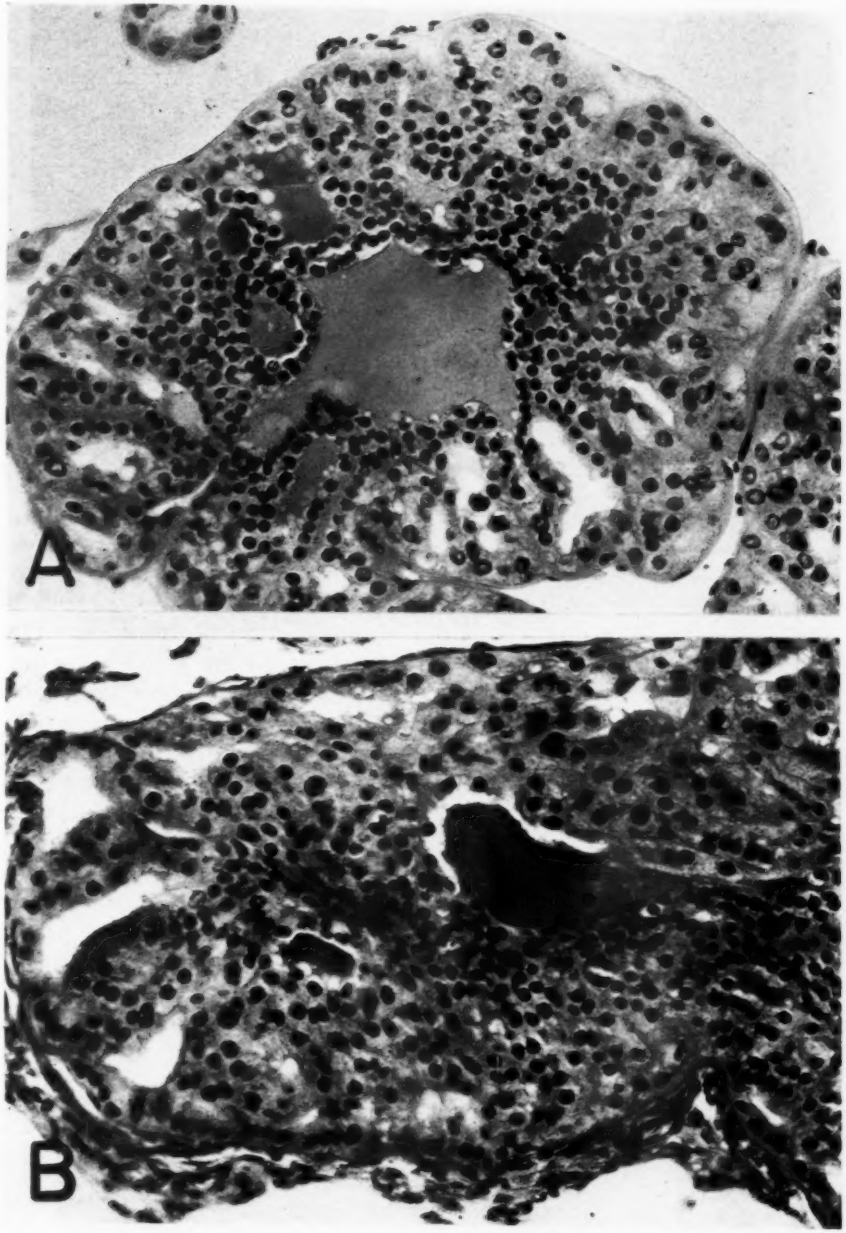


FIGURE 7.—*A*, Thyroid from mouse treated with thiouracil for 464 days, showing reappearance of colloid in some follicles with chromophilic nuclei in the cells lining such follicles. *B*, Pulmonary metastasis of thyroid tissue in the same mouse as in *A*, showing marked similarity to the host thyroid. Both *A* and *B*, Zenker-formol fixation, hematoxylin-eosin stain. × 355.

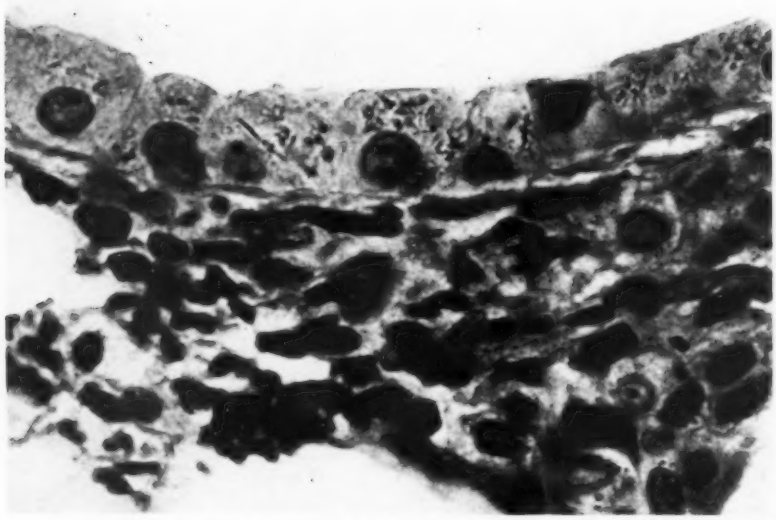


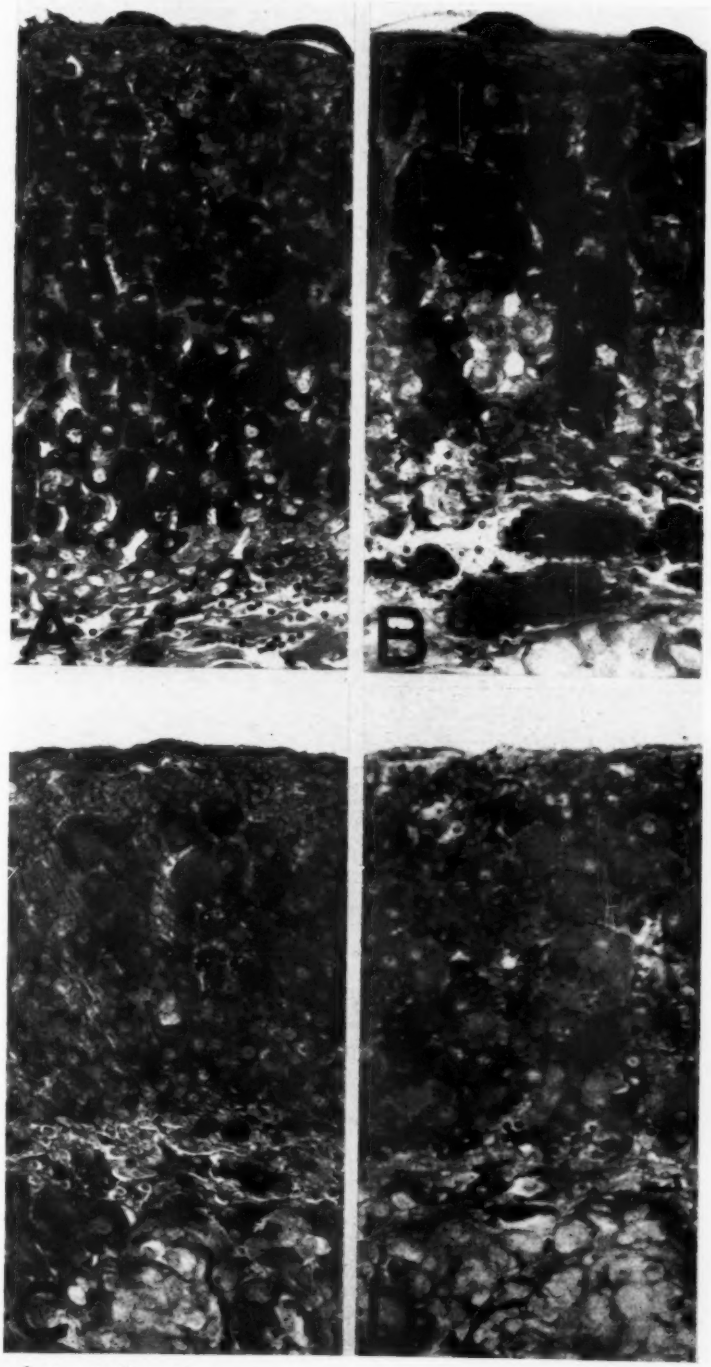
FIGURE 8.—Respiratory epithelium from a bronchiole in the lung of a mouse treated with thiouracil for 464 days, showing pigment in the distal portions of the epithelial cells. Zenker-formol fixation; hematoxylin-eosin stain. × 1040.

droplets. The total thickness of the cortex was approximately the same after 17 weeks as after 3 weeks of thiourea ingestion (fig. 9, *B* and *C*).

After 25 or more weeks of thiourea ingestion, no osmiophilic material was found in the zona fasciculata and very little in the zona glomerulosa. Capsular thickening and the presence of lipid in some of the inner capsule cells were characteristic of animals receiving thiourea for this longer time. There was evidence of a rather rapid loss of fasciculata cells in animals ingesting thiourea for 25 weeks or more. Many cells were shrunken and contained lipid droplets which varied greatly in size and in which the reaction to osmic acid was blue-black rather than the usual dark brown. There was, however, an increase in size of the remaining cells. The final result was a definite decrease in the thickness of the functional cortex (fig. 9, *A* and *D*).

Similar changes were observed in mice ingesting thiouracil. The glands were small (table 1) and consistently showed a decrease in the size and number of osmiophilic droplets when compared with those of controls of the same age. All the control mice eventually developed tumors; and since they died or were killed shortly after tumors appeared, no controls remained for comparison with the last of the experimental group. There was little, if any, further change in the amount and distribution of osmiophilic material after the first year of thiouracil ingestion.

FIGURE 9.—*A*, Control adrenal fixed in Champy fluid and sectioned at 4μ. The cells of the fasciculata are filled with osmiophilic droplets. *B*, Adrenal of an animal fed thiourea for 3 weeks (Champy) showing reduction of osmiophilic droplets in some of the cell cords of the zone fasciculata. *C*, Adrenal of an animal fed thiourea for 17 weeks (Champy), showing depletion of osmiophilic material from the zona fasciculata and its retention by the zona glomerulosa. Note thickened capsule. *D*, Adrenal of animal fed thiourea for 42 weeks (Champy) showing almost complete disappearance of osmiophilic material from both the zona fasciculata and zona glomerulosa. The capsule is thickened, and some capsular cells contain osmiophilic droplets (other than Golgi material). Note enlargement of fasciculata cells. *A*, *B*, *C*, and *D*, × 300.



Legend for FIGURE 9 on opposite page.

Pituitary [4]

In the early stages of thiourea ingestion in mice, no striking changes in the pituitary were noted, such as were described by Purves and Griesbach (*11–12*) for the rat. At no time were the so-called thyroidectomy cells found. In fact, the pituitaries of animals receiving thiourea were characterized by the absence of vacuoles in the cells, whereas control pituitaries regularly showed vacuolization of a small percentage of beta cells. The first change noted was partial degranulation of the majority of alpha cells. This occurred as early as 5 weeks after the beginning of treatment. With continued treatment, this degranulation became more pronounced, and at the end of 42 weeks there remained only a narrow rim of granule-containing cytoplasm surrounding the nucleus of the alpha cells. Along with the decrease in granules, there was a condensation of the Golgi material.

More striking changes were observed in the beta cells, but the changes required longer treatment for their development than did the change in the alpha cells. In the pituitary of control mice of the same age, a majority of the beta cells possessed large, complex, well-defined, heavily impregnated Golgi nets. Following continued treatment with thiourea (17–45 weeks), an occasional beta cell was encountered containing a new type of Golgi net which consisted of fine, tenuous threads arranged in more or less spherical form, with the peripheral portions more heavily impregnated than the more centrally located ones. This type of Golgi net resembled the classic form of the beta-cell Golgi apparatus described by Severinghaus (*13*) for the normal rat. The beta cells with this type of Golgi net became more numerous with continued treatment. In some cases, these cells gave an adenomatoid appearance to the central portion of the anterior lobe, where they tended to accumulate. The extreme degranulation and relative scarcity of the alpha cells at this stage emphasized the adenomalike appearance.

Changes in the pituitaries of mice ingesting thiouracil were similar to those found in mice fed thiourea although they were more extensive and obvious because of the longer periods of ingestion (fig. 10, *A* and *B*). Degranulation of the alpha cells was noted in all 31 pituitaries examined. This was associated with a decrease in the amount of alpha-cell cytoplasm and in the amount of alpha-cell Golgi material (fig. 10, *A*, A, and *B*, A). In pituitaries of mice kept on the thiouracil diet for the longest periods (464–549 days), the alpha cells appeared not only to have decreased in size but also in number. In the two cases in which the pituitaries had an adenomalike appearance, the alpha cells made up a small and relatively insignificant part of the anterior lobe.

The so-called thyroidectomy basophils occurred occasionally in animals fed thiouracil for 12 months or longer. The vacuoles were multiple and small or single and large (fig. 10, *B*, T).

Counts (*14*) of alpha- and beta-cell nuclei, undifferentiated cytoplasm, and Golgi material of 21 control and 25 experimental pituitaries support these observations in that they demonstrate a significant decrease in the amount of alpha-cell cytoplasm and Golgi material and an increase in the amount of beta-cell cytoplasm and Golgi material (table 2). It is interesting that these counts demonstrate no significant change in the amount of alpha-cell nuclear material but do demonstrate a fivefold relative increase in the amount of beta-cell nuclear material, the greatest percentage shift of any of the cellular constituents.

Kidney

No evidence of kidney damage was noted in animals ingesting thiourea for short periods.

In one animal, after 63 weeks on the thiourea diet, the cells of the proximal convoluted tubules were found to possess vacuoles containing peripherally located iron-positive pigment granules. Similar changes were regularly observed in mice receiving thiouracil for long periods. Of 47 mice in this group, 45 contained iron-positive, greenish-brown pigment restricted to the cells of the proximal convoluted tubules. The pigment was usually found in rather large vacuoles frequently located between the nucleus and brush border (fig. 11).

[4] Differential stains for the alpha and beta cells of the mouse pituitary have not given satisfactory results in our hands. Champy fixation followed by 5 days in 2 percent osmic acid at 40° C. has given excellent fixation. In addition, it has allowed us to distinguish between alpha and beta cells by visualizing the differences in the size and configuration of the Golgi material. Following this treatment, the individual secretory granules in alpha cells assume a golden color while those in the beta cells are pale gray to black. The cytoplasm of the chromophobe cells, with the exception of the Golgi material, does not react with osmic acid.

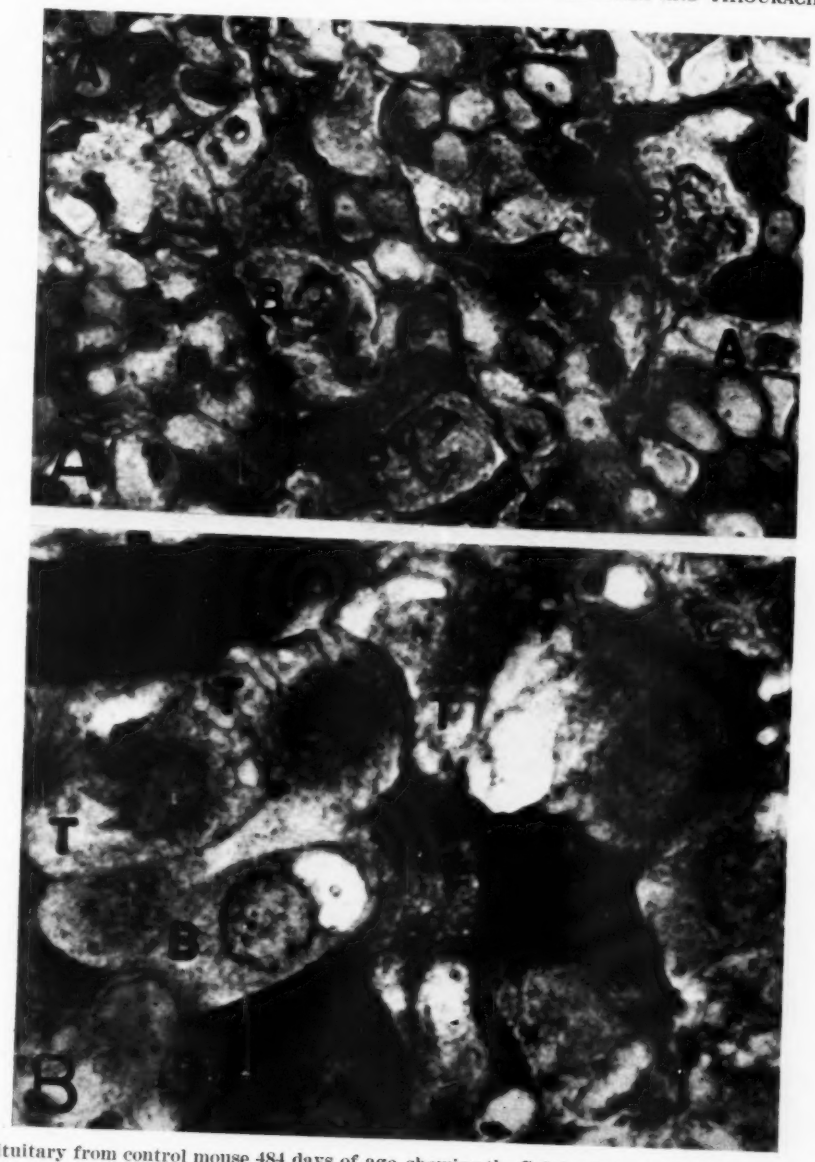


FIGURE 10.—*A*, Pituitary from control mouse 484 days of age, showing the Golgi substance in alpha and beta cells. Alpha cells are small, with dark cytoplasm containing Golgi material in the form of a small, heavily blackened, closely woven net. Beta cells are large, with pale cytoplasm containing Golgi material in the form of a large, open, irregularly spaced network. × 1300. *B*, Pituitary from a mouse treated with thiouracil for 468 days. Alpha cells are smaller than in the control, with less cytoplasm and a small amount of Golgi material. Beta cells are large and darker staining than in the control, with the Golgi material more or less spherical in outline and with the peripheral portions more heavily impregnated than the central. Vacuolated "thyroidectomy" basophils are also present. × 1300. Both *A* and *B* were fixed in Champy and post-osmicated 5 days. (The letter A is placed to the left of alpha cells, B to the left of beta cells, and T to the left of "thyroidectomy" basophils.)

TABLE 2.—*Percentage counts of alpha- and beta-cell components in control and experimental mice*

Group	Number of animals	Alpha cell				Beta cell				Space
		Nucleus	Cytoplasm	Golgi material	Total	Nucleus	Cytoplasm	Golgi material	Total	
		Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Controls	21									
Range		16.5–35.0	25.5–43.5	11.0–33.5	66.5–93.0	0.0–4.5	0.0–15.0	0.5–9.5	2.5–24.5	1.0–11.5
Average		24.9	34.6	20.4	79.9	1.8	7.3	4.3	13.4	6.6
Experimental animals	25									
Range		11.0–31.0	1.0–27.3	1.0–16.5	19.0–65.0	2.0–15.5	18.8–43.5	2.8–16.5	24.5–66.5	5.5–16.0
Average		21.6	9.4	9.5	40.4	9.8	30.6	8.7	49.06	10.8
Significance of difference of average		$P<0.1$	$P>0.01$	$P>0.01$	$P>0.01$	$P>0.01$	$P>0.01$	$P>0.01$	$P>0.01$	$P>0.02$

URINARY BLADDER

Many of the mice maintained on the thiouracil diet for the longer periods developed incontinence. In these animals, stones were found in the urinary bladder. Frequently a single, large, pear-shaped stone was found while in other cases there were five or six small ones. They were green, hard, with a roughened, pitted surface, and when dry could be broken into small sandy particles with moderate pressure. Ten mice, the last of the experimental series, which ingested thiouracil for periods varying from 431 to 549 days, all developed incontinence, and all were found to have bladder stones. The bladder epithelium in the majority of these cases showed the effects of the presence of the stones. Definite keratin formation was not present, but many cells, particularly in the more superficial layers, contained numerous rather large granules staining purple to red with hematoxylin and eosin, similar to the keratohyalin granules found in the granulosa layer of keratinized, stratified, squamous epithelium (fig. 12).

LIVER

Although there was no evidence of liver damage definitely attributable to the thiourea or thiouracil in animals ingesting thiourea for 25 or more weeks and in controls of comparable age, nuclear inclusions were found in many of the hepatic cells

FIGURE 11.—Kidney from mouse treated with thiouracil for 437 days, showing iron-positive pigment lining vacuoles in the cells of the proximal convoluted tubules. Zenker-formol fixation, stained for iron. × 1300.

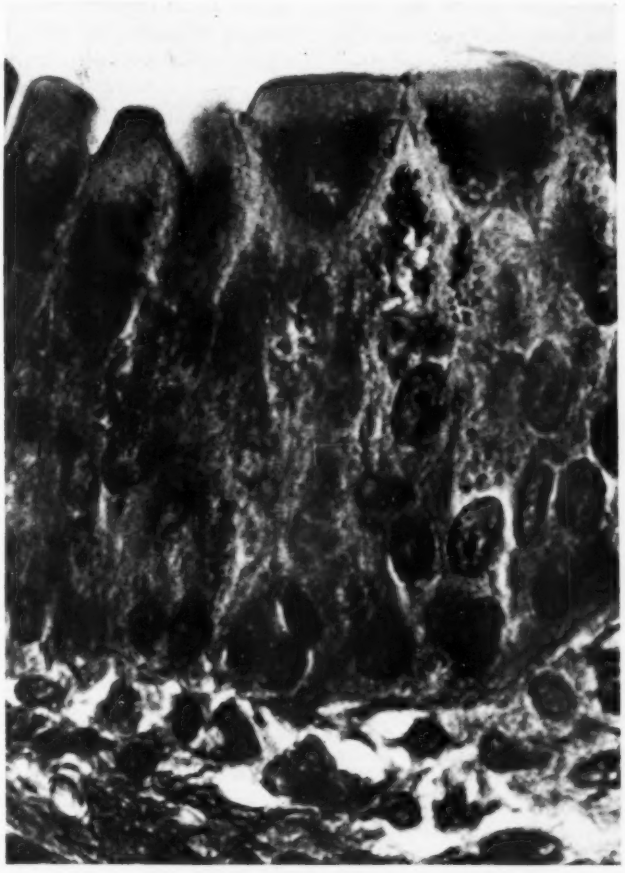


FIGURE 12.—Mucosa of urinary bladder from a mouse treated with thiouracil for 468 days, showing the keratohyalin granules in the more superficial cells of the epithelium. Zenker-formol fixation; hematoxylin-eosin stain. × 1300.

immediately surrounding the central vein. These inclusions were also seen occasionally in cells at a distance from the central vein. Since these mice ranged from 12 to 26 months in age, this observation supports the view of others (*15*, *16*) that these inclusions are associated in some way with aging.

The only difference noted between the livers of mice fed thiouracil and those of the controls was the presence of small amounts of a diffuse, iron-positive, pale-yellow pigment in 16 of the 45 livers. A spontaneous hepatoma was identified in the liver of an animal fed thiouracil for 444 days.

OVARY

Histologic preparations of ovaries from seven animals treated with thiourea for 13 weeks or less resembled those from control animals. There were apparently active corpora lutea, and three of the seven contained large Graafian follicles. Of seven mice ingesting thiourea for 20 weeks or more, only one showed an apparently active corpus luteum, and none contained large Graafian follicles.

There were more degenerating ova in the ovaries of thiourea-fed mice than in the controls (five controls showed a few such ova), and there was a relative increase in the number of immature Graafian follicles and ova showing degenerative changes. Degenerative changes in ova were evidenced by the condensation of the germinal vesicle and the greatly increased eosinophilia of the cytoplasm of these cells. Degenerative changes in the immature

Graafian follicles were evidenced by the fact that the nuclei of the zona granulosa showed pyknosis and the cytoplasm showed hazy, ragged margins.

Thiouracil produced changes in the ovaries comparable with those caused by thiourea (fig. 13, *A* and *B*).

Uterus

The effect of feeding thiourea was not so clear-cut in the uterine endometrium as in the ovary. The uteri of animals ingesting thiourea and thiouracil were visibly smaller (cf. table 1), and the endometrium showed fewer and less complex glands than did those of the controls (fig. 14, *A* and *B*). However, in only 4 of 17 animals receiving thiourea was there histologic evidence of a wholly inactive epithelium. In all others there was some evidence of stimulation, 2 showed secretory activity, 3 leukocytic infiltration, and 8 mitotic activity in varying degree. These histologic changes were in contrast with the evidence of stimulation characteristic of the controls, in which all showed secretory activity, mitosis, or leukocytic infiltration.

Figure 14 shows the relatively inactive epithelium in the uteri of mice fed thiouracil. An exception to this usual picture was found in one

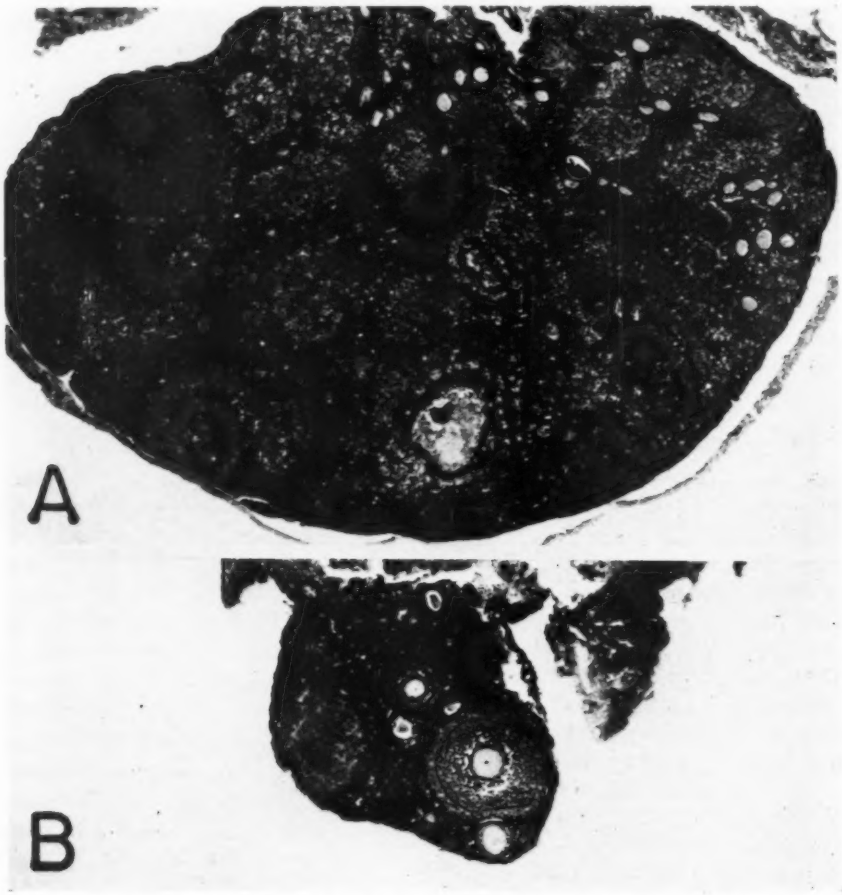


Figure 13.—*A*, Ovary from a control mouse 352 days of age, showing immature ova, follicles, corpora lutea, and interstitial cells. × 55. *B*, Ovary from a mouse treated with thiouracil for 464 days, showing immature ova and developing follicles, but no corpora lutea or interstitial cells. × 65. Both *A* and *B* fixed in Zenker-formol and stained with hematoxylin and eosin.

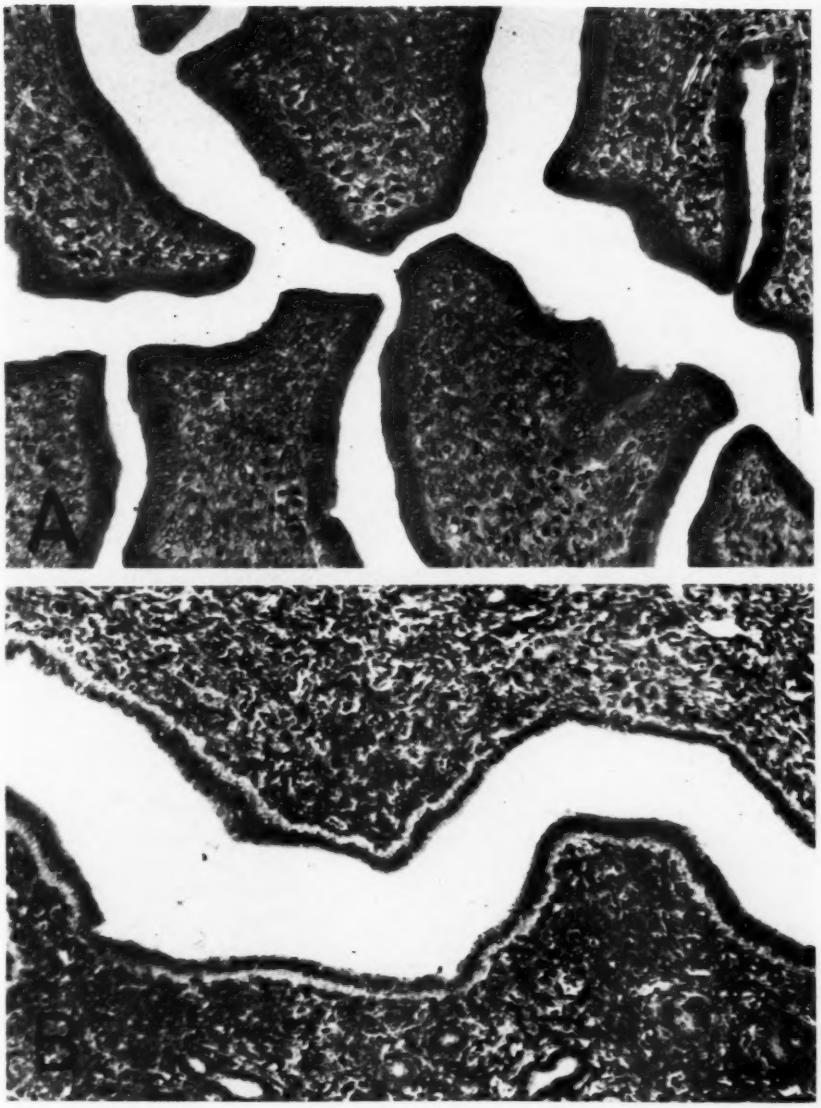


FIGURE 14.—*A*, Endometrium of the uterus of a control mouse 412 days of age. *B*, Endometrium of mouse treated with thiouracil for 440 days, showing a thin, inactive epithelium and relatively few, shallow glands. Both *A* and *B*, Zenker-formol fixation; hematoxylin-eosin stain. × 200.

animal in which the uterus and ovaries combined weighed 110 mg. The ovaries contained numerous interstitial cells. Although this animal had ingested thiouracil for 370 days, its thyroid weighed only 12.3 mg., less than that of any other mouse in the experimental series.

Spleen

While the weights of the spleen, lymph nodes, and thymus were not taken, these organs appeared on simple visual inspection to be smaller in the experimental mice of both groups than in controls of the same age, but there was no detectable difference in the gross or histologic appearance of the red pulp. In the white pulp of the controls, there was relatively little iron-positive pigment, and here an increase in pigment in the spleens of the experimental animals was fairly obvious (fig. 15, *A* and *B*).

In 21 of 37 spleens of the experimental group, there was a relative increase in the amount of erythropoietic tissue in the red pulp when compared with spleens from control animals. This increase was obvious even in instances in which numerous red blood corpuscles were in the red pulp.

Vascular Damage

A vascular lesion developed in animals ingesting both thiourea and thiouracil. The lesion consisted of a laminated deposit, usually between the elastica interna and the endothelium, occasionally in the tunica media. Usually small arteries were the vessels involved although instances of the involvement of venules and small veins were noted. Since this vascular change was first noted in the thyroid (fig. 16) and kidneys of animals fed thiourea, it was expected that many of the thyroids in the thiouracil series would show this change. Actually only 13 examples were found in the 50 thyroids examined, whereas it was found in 18 of 37 cases in the pancreas, and in 31 of 45 kidneys.

Vascular damage when present was without exception in the small arteries of the pancreas. The lesions, while not obvious on gross inspection, frequently occupied large areas of the vessel wall and caused partial occlusion (fig. 17). One such lesion was found which appeared to be calcified.

The vascular lesions were particularly numerous in the small arteries in the kidney. The majority showed the picture characteristic of the lesion in the vessels of other organs, but these laminated deposits frequently appeared to be calcified (fig. 18).

Mammary Glands and Mammary Tumors

There was no evidence of any difference in the general morphology or structural detail of the tumors of treated mice when compared with those of the controls. The involutionary changes in the mammary gland previously described from studies of whole mounts (*6*) could not be detected by the histologic method.

DISCUSSION

The prolonged ingestion of thiouracil obviously resulted in cell and organ changes in many parts of the organism. As discussed herein, the thyroid hyperplasia, pulmonary metastases of thyroid tissue, and the shift in the cell population of the pituitary may be considered the direct result of reduced thyroxin output. The changes produced in the uterus, ovaries, and adrenal glands may be considered indirect in that they are secondary to the reduction in thyroxin. The appearance of pigment in the respiratory epithelium and kidney, the formation of stones in the urinary bladder, and the development of vascular lesions may be considered side effects similar to the induction of leukopenia in human beings.

Thyroid

The histologic changes in the thyroid glands of rats on goitrogenic diets for relatively short periods were previously described by others (*1, 17*). The change in the follicular colloid from acidophilic to faintly basophilic in its staining reaction occurred very soon after the mice were placed on the experimental diet. The collapse of the follicles, eventual loss of colloid, hypertrophy of the individual epithelial cells from cuboidal to columnar, with increase in size and decrease in basophilia of their nuclei, were all observed in this study. Significant deviations from this picture were seen only after long periods of ingestion of thiouracil. These changes consisted of a localized reappearance of acidophilic colloid in certain follicles, with a decrease in size and an increased basophilia of the nuclei of the cells lining such fol-

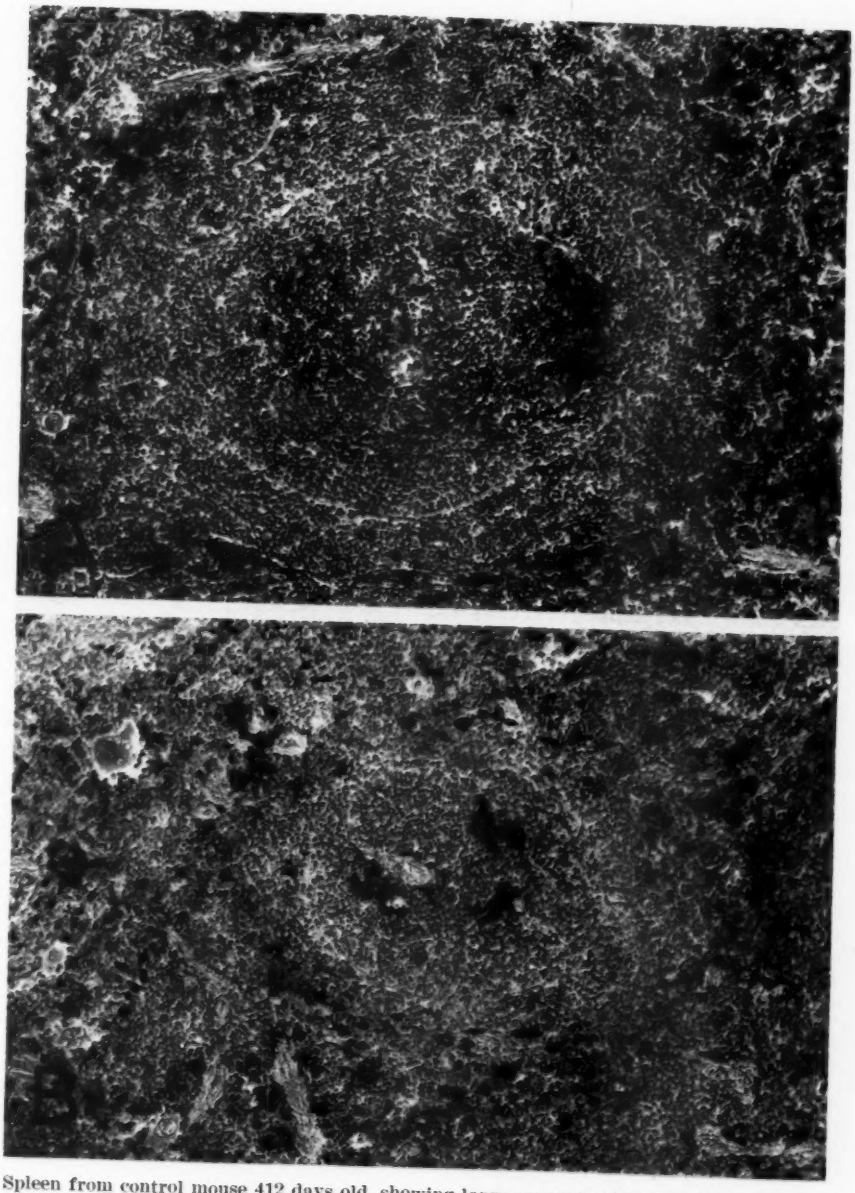


FIGURE 15.—*A*, Spleen from control mouse 412 days old, showing large amounts of iron-positive pigment in the red pulp and a small amount in the splenic nodule. × 155. *B*, Spleen from mouse treated with thiouracil for 411 days, showing similar amounts of pigment in the red pulp as in *A* but larger amounts in the splenic nodule. × 155. Note the smaller size of splenic nodule in *B* as compared with that in *A*.

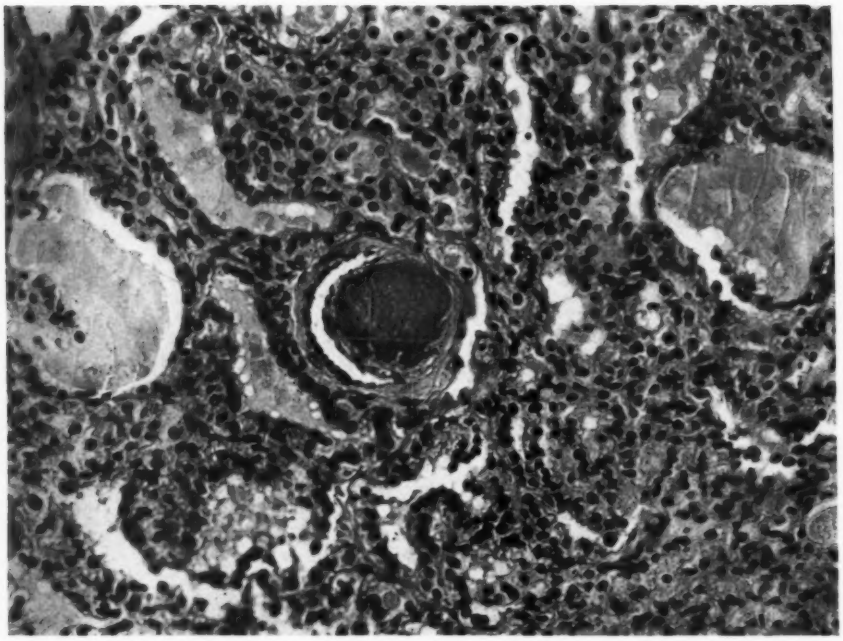


FIGURE 16.—Thyroid from mouse 89 weeks old, killed after 45 weeks of thiourea ingestion, showing hyaline material accumulated in the media of a small blood vessel. Zenker-formol fixation; hematoxylin-eosin stain. × 300.

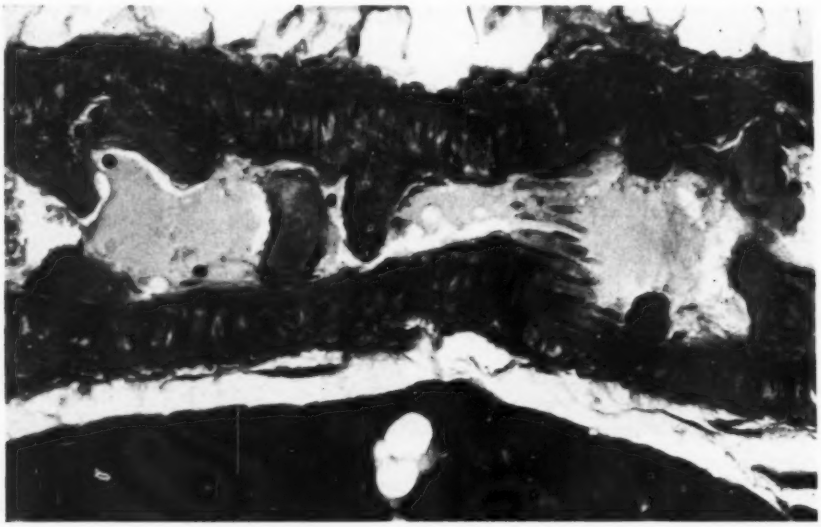


FIGURE 17.—Longitudinal section of small artery in the pancreas of a mouse treated with thiouracil for 301 days, showing the numerous laminated, hyaline lesions of the intima. Zenker-formol fixation; hematozylin-eosin stain. × 355.

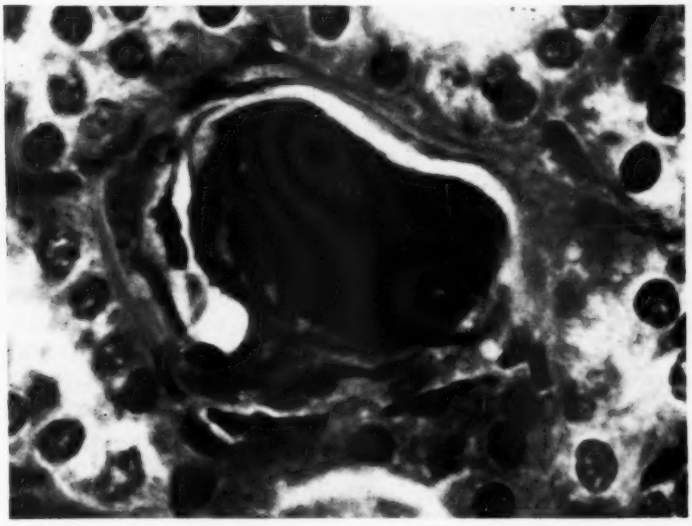


FIGURE 18.—Kidney from mouse treated with thiouracil for 431 days, showing a vascular lesion in an arteriole which appears to be calcified. Zenker-formol fixation; hematoxylin-eosin stain. × 1300.

licles, changes which are suggestive of a return toward the picture characteristic of the normal mouse thyroid. They were noted during the period when there was the greatest hypertrophy and greatest variation in thyroid weight and when pulmonary metastases of thyroid tissue were found.

Three possibilities suggest themselves with respect to the cause of the reversal toward the normal histologic appearance: (1) The animal as a whole is no longer capable of reacting completely to thiouracil; (2) the thyroid gland itself is no longer capable of responding to stimulation by the pituitary; and (3) the pituitary is no longer producing the amount of thyrotropic hormone necessary to maintain the thyroid in a hyperplastic state. Evidence from the growth and histology of thyroid tissue in transplants, both from the thyroid gland itself and from metastatic nodules, indicates that thyroid tissue is capable of responding to pituitary stimulation for periods well beyond the life span of the mouse.[5] This fact suggests that either the organism as a whole or the pituitary gland alone becomes refractory to a stimulus which originally and for a period of approximately 12 months thereafter maintained the thyroid in a hyperplastic state.

[5] Morris, H. P., and Dubnik, C. S.: Unpublished data.

The question of the significance of the "parafollicular" cell was considered by Altmann (*18*) and more recently by Thomas (*19*). The cells which we have tentatively identified as "parafollicular" are similar to those described by Thomas in the thyroids of rats treated with thiourea. The fact that in his material the nuclei were not pyknotic but were in ours may possibly be explained by the longer treatment received by our mice. These cells have never been found in thyroids of normal animals and appear to be characteristic of thyroids maintained in a condition of chronic hyperplasia. They were usually parafollicular in position in mice fed thiourea; they were much more numerous in mice receiving thiouracil for the longer periods, and in these cases they nearly always remained in their original position. Thus the term "pale cells of the thyroid" more accurately describes them and is noncommittal with regard to location.

In searching for an explanation of the appearance of "parafollicular" or "pale" cells during the administration of thiourea, it is not necessary to assume, as Thomas does, that thiourea effects primarily an alteration in the permeability of the thyroid-cell membrane. It is suggested instead that the presence of such cells is an indication of long-continued stimulation and that these cells

are on the verge of degeneration. The possibility of this supposition is supported by the fact that the nuclei of the "pale" cells are almost always pyknotic. A change in the permeability of the thyroid-cell membrane may well be the result of long-continued stimulation and approaching exhaustion and death, rather than being the primary factor which results in their formation.

From the cytologic appearance of the majority of thyroid cells, it is obvious that thiourea ingestion has stimulated them to excessive activity; yet in spite of this, the formation of thyroglobin is prevented. It remains for future study to determine what substances, if any, are the products of this apparent activity.

Pituitary

The changes observed in the pituitary were in a general way similar to those observed in the rat by Reese, Koneff, and Wainman (*20*) and Zwecker et al. (*21*) following thyroidectomy, and by Griesbach (*17*) after feeding a goitrogenic diet. In our study, alpha cells were found to contain fewer granules in the experimental animals than in the control, and in some instances almost complete absence of granules was noted. This is in contrast with the statement of Griesbach and Purves (*11*):

> Thiourea administration alone never causes extensive degranulation of pituitary acidophil cells, and in this respect the action of thiourea resembles the effect of subtotal thyroidectomy rather than that of the total operation.

Purves and Griesbach later (*12*) reported that giving thiourea plus iodide to intact rats results in full thyroidectomy changes in the pituitary. Apparently the structural changes induced in pituitary cells by goitrogenic diets are in some respects species-specific and are also influenced by the iodide content of the diet. This is further emphasized by the fact that true thyroidectomy basophils were found in our study only after very long periods of ingestion of thiouracil and then only occasionally. In fact, one of the characteristics of the pituitaries from thiourea-treated animals was the absence of vacuoles, particluarly in those from animals treated for 25 weeks or longer. This was in contrast with the controls, in which the beta cells frequently contained one to many large, strongly osmiophilic droplets.

The type of Golgi net in the beta cell of the pituitary of the normal C3H mouse was found to be different from the type originally described by Severinghaus (*13*) for the rat. Not until treatment was continued for some time and hypertrophy of the beta cells had taken place did the distribution of the Golgi substance come to resemble that in the beta cells of the normal rat pituitary.

The longer interval of ingestion and the larger number of mice used in the thiouracil experiment allowed us to make a more accurate analysis of the cytologic changes in the anterior pituitary than was possible in the study with thiourea.

Analysis of the counts (*14*) made on alpha- and beta-cell nuclei, cytoplasm, and Golgi material clearly shows a relative increase in beta-cell over alpha-cell material. While there is a significant increase in beta-cell nuclei, the major part of the shift comes from an increase in beta-cell Golgi substance and undifferentiated cytoplasm and a decrease in the amount of these components in the alpha cells. If, as seems likely, the beta cells are responsible for the elaboration of thyrotropic hormone, then this would be an example of increase in size of the active components (beta cells) and a decrease in size (loss of granulation) of the inactive components (alpha cells) of a gland as a result of long-continued stimulation. This is in contrast with the adrenal, in which storage takes place during inactivity, with loss of lipid and eventual death of cells from long-continued, excessive stimulation.

Apparently the pituitary of the mouse reacts to goitrogenic diets somewhat differently from that of the rat (*22*). In this study, only occasional thyroidectomy basophils were found, yet there was a marked decrease in alpha-cell cytoplasm. In the rat, Griesbach and Purves (*11*, *12*) reported that thyroidectomy basophils were numerous following similar treatment with goitrogens, and in addition the alpha cells apparently did not show the degree of degranulation noted in the present study.

Adrenals

Loss of lipid from the adrenals is usually considered to result from stimulation by adrenocorticotropic hormone of the pituitary since it does not occur in hypophysectomized animals and may be

induced with relatively pure adrenocorticotropic hormone (*23*). Excessive storage of lipid is known to be present in hypophysectomized animals and also following administration of excessive amounts of adrenal cortical hormone or of potent adrenal cortical extract.

It is possible that the adrenal changes observed in our experiment may have resulted from long-continued and excessive stimulation by adrenocorticotropic hormone from the pituitary. This view is supported by the evidence of excessive numbers of degenerating cells and cellular debris at the corticomedullary border, suggesting exhaustion and death of cells, due perhaps to long-continued and excessive stimulation. Kennedy and Purves (*17*) noted lipid storage in and hypertrophy of the adrenal cortex in rats fed a goitrogenic diet for relatively short periods. Thus it appears that thiourea and thiouracil not only stimulate the production of thyrotropic hormone but also may either directly or indirectly stimulate an increase in the production of adrenocorticotropic hormone in the pituitary. The changes in the adrenal cortex described by Baumann and Marine (*4*) and ascribed by them to "involution" may, therefore, be the result of overactivity rather than inactivity.

Uterus and Ovaries

Since both experimental and control animals were 11 months of age at the start of the thiourea experiment, the effect of long-continued feeding of this goitrogen on the reproductive system was not so clear-cut as it was in the thiouracil experiment in which younger animals were used. In general, however, in the thiourea group there was a decrease in the large follicles and an increase in degenerating ova in the ovaries and the presence of inactive endometria in the uteri of the experimental mice. The general picture of decreasing activity of the ovaries is supported by the absence of regular estrus cycles in the majority of animals, as demonstrated by the vaginal-smear technique.

The histology of the uterus and ovaries of the thiouracil group was similar to that of normal immature mice of the same strain and probably resulted from a low gonadotropic hormone output by the pituitary. Whether this in turn was the result of hormone imbalance or partial inanition, or a combination of these two factors, is still unsettled. Unpublished data of Morris and Dubnik from replacement therapy studies suggest the former as the most likely. The reduction in thyroxin output resulting from the ingestion of goitrogens stimulated an excess secretion of thyrotropic hormone by the pituitary, which in turn resulted in extreme thyroid hyperplasia. Apparently a concomitant and correlated decrease in gonadotropic hormone output took place, resulting in the failure of the ovaries and uterus to develop in immature mice and in their atrophy in adult mice.

Pulmonary Metastases

Gorbman (*24*) demonstrated the presence of thyroid tissue in blood vessels of thyroids of mice fed thiouracil. We found similar evidence in our material. This, together with the presence of endothelium surrounding the nodules of thyroid tissue in the lungs of thiouracil-treated animals, suggests that the pulmonary nodules are true metastases from the host thyroid gland and do not represent thyroid rests. The chance that during development as many as 10 to 15 separate pieces of potential thyroid tissue could be carried from their place of origin at the base of the tongue to the superficial pleural surfaces of the various lobes of the lung seems rather slight.

In these 10 cases of pulmonary metastases of thyroid tissue, no histologic evidence of neoplasia was found in the host thyroids. Apparently these are cases of "benign metastasizing" thyroid tissue.

Pigmentary Changes

The pigment in the epithelial cells of the thyroid is iron-positive. It was found in all cases where hemorrhage into follicles was present. It seems reasonable to assume that all iron-positive pigment in thyroid cells appeared following hemorrhage into the follicles and was the result of absorption of hemoglobin or hemosiderin by the follicle cells from the lumen of the follicles.

The increase in iron-positive pigment in the spleen and liver and its appearance in the cells of the proximal convoluted tubules of the kidney are apparently related phenomena and are possible

evidence of the toxic action of thiouracil on red blood corpuscles. The appearance of iron-positive pigment in the kidney cells can best be explained by an increase in the amount of free hemoglobin in the blood. The few tests made for free hemoglobin suggest that this increase does occur. This increase could result either from an increased destruction of red blood corpuscles in the spleen or from breakdown of red cells in the blood stream.

It is possible that there is some causal relationship between the presence of pigment in the respiratory epithelium and the urinary bladder stones. Since, in our experience, these substances were found only in animals fed thiouracil, it is possible that they were deposited as insoluble products of the metabolism of this drug.

Vascular Damage

The vascular damage is of particular interest. Altschul and Brown (*25*) described similar changes in the arteries of the parathyroids, thyroid, and viscera in a case of Wilson's disease. They found no evidence of calcification of the lesions, such as appeared to occur occasionally in our material. However, the laminated appearance, the slightly basophilic staining reaction, and the usual location near the internal elastic membrane of small arteries are characteristic of both lesions, so that there is more than superficial resemblance between the two. Although Wilson's disease is usually characterized by liver necrosis and damage to the central nervous system, there was no evidence of either in mice fed thiouracil for the longest periods. One explanation that could relate the two conditions would be that they are the expression of direct toxic effects on the intima of blood vessels, in mice from the direct toxic action of thiouracil, in man from toxic materials released into the general circulation following chronic severe liver necrosis.

SUMMARY

Organs of female C3H mice were examined histologically and cytologically to ascertain the effects of long-continued ingestion of thiourea and thiouracil.

Changes were found in the adrenals, pituitary, ovary, uterus, kidney, pancreas, spleen, lungs, and blood vessels, as well as in the thyroid. The adrenals showed changes which could have resulted from overstimulation and might not be involutionary in character.

Pituitary changes consisted in a degranulation of acidophils, with a decrease in size and number of alpha cells. Large beta cells in greater numbers and with a change in form of the Golgi material were found. It was concluded that "thyroidectomy basophils" were not, under our experimental conditions, characteristic of the pituitary glands of adult mice fed thiourea. These cells were found occasionally in mice fed thiouracil for longer periods.

The effects on the reproductive system were suggestive of a decrease in pituitary gonadotropin, with cessation of estrus in adult mice fed thiourea and failure of development of ovaries and uterus in those fed thiouracil.

Iron-positive pigment in the proximal convoluted tubules of the kidney and an increase in the amount of pigment in the spleen were noted.

Vascular degeneration consisting of laminated deposits in the intima and inner media of small arteries was found in the thyroid, kidneys, and pancreas. The significance of this lesion is not known.

Changes in the thyroid are described, and the significance of the "parafollicular" or "pale" cells is discussed. Pulmonary metastases of thyroid tissue were found in animals fed thiouracil for long intervals. These may possibly be considered cases of "benign metastasizing thyroid tissue."

REFERENCES

(1) MACKENZIE, J. B., and MACKENZIE, C. G.: Effect of "sulfa" drugs on the thyroid glands in rats and mice. Federation Proc. 1: 122–123 (1942).

(2) MACKENZIE, J. B., and MACKENZIE, C. G.: Effect of prolonged and intermittent sulfonamide feeding on the basal metabolic rate, thyroid and pituitary. Bull. Johns Hopkins Hosp. 44: 85–97 (1944).

(3) WILLIAMS, R. H., WIENGLASS, A. R., BISSELL, G. W., and PETERS, J. B.: Anatomical effects of thiouracil. Endocrinology 34: 317–328 (1944).

(4) BAUMANN, E. J., and MARINE, D.: Involution of the adrenal cortex in rats fed with thiouracil. Endocrinology 36: 400–405 (1945).

(5) MARINE, D., and BAUMANN, E. J.: Periarteritis nodosa-like lesions in rats fed thiouracil. Arch. Path. 39: 325–330 (1945).

(6) MORRIS, H. P., DUBNIK, C. S., and DALTON, A. J.: Effect of prolonged ingestion of thiourea on mammary glands and the appearance of mammary tumors in adult C3H mice. J. Nat. Cancer Inst. 7: 159–169 (1946).

(7) DALTON, A. J., MORRIS, H. P., and DUBNIK, C. S.: Changes in the organs of female C3H mice receiving thiourea. J. Nat. Cancer Inst. 5: 451–454 (1945).

(8) DALTON, A. J., MORRIS, H. P., and DUBNIK, C. S.: Changes in the thyroid and other organs in mice receiving thiouracil. Federation Proc. 5: 219 (1946).

(9) MORRIS, H. P., DUBNIK, C. S., and DALTON, A. J.: Mammary tumor incidence and occurrence of growths of thyroid tissue in the lungs of mice after prolonged ingestion of thiourea and thiouracil. Cancer Research 6: 492 (1946).

(10) MALLORY, F. B., and WRIGHT, J. H.: Pathological technique. Ed. 8, p. 207. W. B. Saunders Co., Philadelphia (1924).

(11) GRIESBACH, W E., and PURVES, H. D.: Significance of the basophil changes in the pituitary accompanying various forms of thyroxine deficiency. Brit. J. Exper. Path. 26: 13–17 (1945).

(12) PURVES, H. D., and GRIESBACH, W. E.: Observations on the acidophil cell changes in the pituitary in thyroxin deficient states. I. Acidophil degranulation in relation to goitrogenic agents and extra thyroidal thyroxine synthesis. Brit. J. Exper. Path. 27: 170–179 (1946).

(13) SEVERINGHAUS, A. E.: Cytological study of anterior pituitary of rat, with special reference to Golgi apparatus and cell relationship. Anat. Rec. 57: 149–175 (1933).

(14) CHALKLEY, H. W.: Method for the quantitative morphologic analysis of tissues. J. Nat. Cancer Inst. 4: 47–53 (1943).

(15) FINDLAY, G. M.: Intranuclear bodies in the liver cells of mice. Brit. J. Exper. Path. 13: 223–229 (1932).

(16) ANDREWS, W., BROWN, H. M., and JOHNSON, J. B.: Senile changes in liver of mouse and man with special reference to similarity of nuclear alterations. Am. J. Anat. 72: 199–221 (1943).

(17) KENNEDY, T. H., and PURVES, H. D.: Studies on experimental goitre. I. The effect of Brassica seed diets on rats. Brit. J. Exper. Path. 22: 241–244 (1941).

(18) ALTMANN, H. W.: Die Parafollikuläre Zelle der Schilddrüse und ihre Beziehungen zu der gelben Zelle des Darmes. Beitr. z. path. Anat. u. z. allg. Path. 104: 420–454 (1940).

(19) THOMAS, O. L.: A cytological study of the secretion antecedents of rat thyroids activated by thiourea. Anat. Rec. 89: 461–473 (1944).

(20) REESE, J. D., KONEFF, A. A., and WAINMAN, P.: Cytological differences between castration and thyroidectomy basophils in the rat hypophysis. *In* Essays in Biology, pp. 47–485. Univ. California Press (1943).

(21) ZWECKER, I. T., DAVISON, L. W., KELLER, T. B., and LIVINGOOD, C. S., JR.: Pituitary in experimental cretinism. I. Structural changes in the pituitaries of thyroidectomized rats. Am. J. M. Sc. 190: 145–157 (1935).

(22) GRIESBACH, W. E.: Studies on experimental goitre. II. Changes in the anterior pituitary of the rat produced by Brassica seed diet. Brit. J. Exper. Path. 22: 245–249 (1941).

(23) SAYERS, G., SAYERS, M. A., FRY, E. G., WHITE, A., and LONG, C. H. H.: Effect of the adrenotrophic hormone of the anterior pituitary on the cholesterol content of the adrenals, with review of literature of adrenal cholesterol. Yale J. Biol. & Med. 16: 361–392 (1944).

(24) GORBMAN, A.: Thyroid changes induced by prolonged feeding of thiourea. Cancer Research 6: 492 (1946).

(25) ALTSCHUL, R., and BROWN, J. S.: Wilson's disease. Canad. M. A. J. 51: 436–438 (1944).

TWO TRANSPLANTABLE RAT CARCINOMAS INDUCED WITH N-ACETYL-2-AMINOFLUORENE[1]

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INTRODUCTION

The discovery by Wilson, De Eds, and Cox (*1*) of an unusually versatile carcinogen, N-acetyl-2-aminofluorene, which in the rat produces a variety of neoplasms at sites distant from the point of entry suggested new possibilities for the study of carcinogenesis. In this laboratory during a study of the transplantability of a number of neoplasms induced by this agent, new and useful transplantable rat tumors have been obtained. Since transplantable rat tumors have been difficult to obtain and some of the tumors developed in this laboratory have been used by other investigators at this Institute, two such transplantable tumors are described.

MATERIALS AND METHODS

The two tumors were induced in the offspring of rats obtained originally from the Institute of Malignant Disease, Buffalo, N. Y., about 1931. This strain, designated Buffalo, is an albino similar in size to the Wistar albino, and it probably originated from Wistar stock. The strain is not yet highly inbred because close inbreeding was not practiced prior to 1943.

Pieces from the original tumors and all transplant generations were fixed in Zenker-formol, embedded in paraffin, and sectioned. Hematoxylin-eosin stains were made routinely; and special procedures were used for the study of the original tumors and the first few generations. These procedures were: phosphotungstic acid-hematoxylin for demonstration of cytoplasmic fibrillae, Van Gieson for demonstration of collagen, Wilder method of silver impregnation for demonstration of reticulum, toluidine blue for metachromatic staining of mast cells, and a method for demonstrating mucin and glycogen by means of Schiff's reagent after action by periodic acid. This last method gives a red color to glycogen and mucin. The glycogen can be removed by diastase digestion, and this procedure thus differentiates these two substances.

[1] Received for publication July 1, 1948.

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GROSS PATHOLOGY OF TUMOR 2226

Tumor 2226 was induced in a rat receiving a diet containing 0.05 percent of N-acetyl-2-aminofluorene and 0.25 percent thiouracil for 28 weeks. A protusion of the left eye was noted in this rat 2 weeks after cessation of feeding the carcinogen-containing diet. One week later, the eye apparently having been destroyed by the neoplasm, the rat was killed and examined.

A small primary tumor about 1 cm. in diameter was found in the orbital cavity. The eye was dissected free of the tumor, which occupied the posterior portion of the orbital cavity, where it was closely attached to the Harderian gland and extended forward to involve the eyelid. Grossly the tumor consisted of soft pink tissue without necrosis.

Young Buffalo rats were inoculated subcutaneously with this primary tumor on November 5, 1945. A tumor was palpated 7–10 days after inoculation, growth was rapid, and new transplants were made after 4 weeks. Over a period of 116 weeks, 31 transplants were made, an average of 3.7 weeks for each transplant generation. The percentage of takes was always over 50 percent, and in the last 12 generations all inoculated animals developed tumors. This tumor also grows readily in Marshall strain rats.

The tumor grows readily when injected subcutaneously, intramuscularly, or intraperitoneally. When inoculated intraperitoneally, the tumor implants widely on visceral and parietal peritoneum and grows and occasionally spreads through the diaphragm into the thoracic cavity invading the lungs and thoracic wall. Numerous metastases to the lungs also occur after subcutaneous inoculation.

MICROSCOPIC PATHOLOGY OF TUMOR 2226

Microscopically, the primary tumor involved the Harderian gland and may have originated in this tissue although there was no close histologic resemblance to the acinar cells of this gland. The tumor cells were large, rounded, hyperchromatic, and uniform (fig. 1). An indication of gland

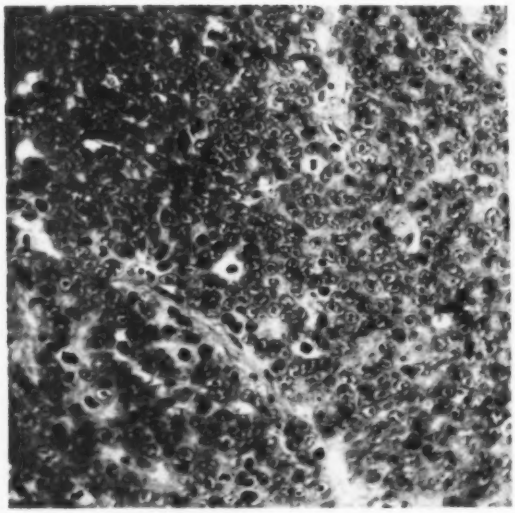

FIGURE 1.—Carcinoma 2226, a very cellular tumor with frequent mitotic figures and abortive gland formation. Hematoxylin-eosin stain × 300.

formation was seen in some areas where low cuboidal cells were grouped around lumina. Mitotic figures were extremely numerous (five–seven per high-power field).

A considerable difference was found in the amount of stroma in various tumors from succeeding generations. In many sections, broad bands of connective tissue separated groups of tumor cells, while in other sections the tumor cells were closely packed. Regardless of the stromal pattern, the tumor cells remained uniform. The nuclei were round, vesicular, and usually contained a single prominent nucleolus. Silver impregnation by the Wilder method showed a separation by reticulum fibers into clumps of cells, following a carcinomatous pattern. Periodic acid stains showed no red staining material within the tumor cell cytoplasm. In a frozen section of the original tumor, no sudanophilic substance was found in tumor cells, although necrotic portions of the tumor contained sudanophilic granules. Considering the rapidity of growth of this tumor strain, relatively little necrosis developed in this tumor, and there were no hemorrhages or inflammatory reactions associated with the tumor growth. Vascular channels were found within the connective tissue bands and were not in close association with tumor cells. This tumor was diagnosed a carcinoma.

GROSS PATHOLOGY OF TUMOR 1643

Tumor 1643 arose in a rat fed 2-acetylaminofluorene dissolved in coconut oil administered via stomach tube at a dose of 2.7 mg. per 100 gm. body weight, six times weekly for 18 weeks. During the twenty-sixth week of the experiment a mass was observed in the region of the left groin which slowly increased in size for 9 weeks before necropsy on March 8, 1946.[2]

The tumor was inoculated subcutaneously into 4 young Buffalo-strain rats, and after 11 weeks 2 rats developed tumors. The tumor was carried through 12 generations during 108 weeks, an average of about 9 weeks for each generation.

This tumor grows after subcutaneous, intraperitoneal, or intramuscular inoculation.

Routinely each animal received both an intramuscular and an intraperitoneal inoculation. For the first eight generations, the tumor growth always remained localized at the site of inoculation. In one animal bearing a ninth-generation transplant, the intraperitoneal transplant instead of remaining localized as a single mass spread throughout the abdominal and thoracic cavities. Tenth-generation transplants were made from this animal and also from one animal which still had a single intraperitoneal transplant. In subsequent generations, however, the lines derived from the two sources behaved similarly by spreading throughout the animal. The locations of the metastases suggest that the tumor probably spreads through the lymph channels. It grows in flat sheets over the omentum and always invades the thymus. No metastases to the lungs were observed after subcutaneous inoculation.

MICROSCOPIC PATHOLOGY OF TUMOR 1643

Microscopically, the original tumor (fig. 2, *A*) was considered to be a mammary carcinoma with acinus formation, although there were a few areas in which the epithelial cells seemed to merge with the stroma. In the second and third generations, the acinous appearance of the tumor was less evident (fig. 2, *B*). Most of the cells were arranged in double or single rows suggesting an epithelial pattern although lumina were indistinct

[2] In addition to the subcutaneous tumor of the groin, this rat had a hyperkeratotic lesion of the auditory canal, which was considered to be a precancerous lesion, and a carcinoma of the uterus. The uterine tumor was transplanted but failed to grow.

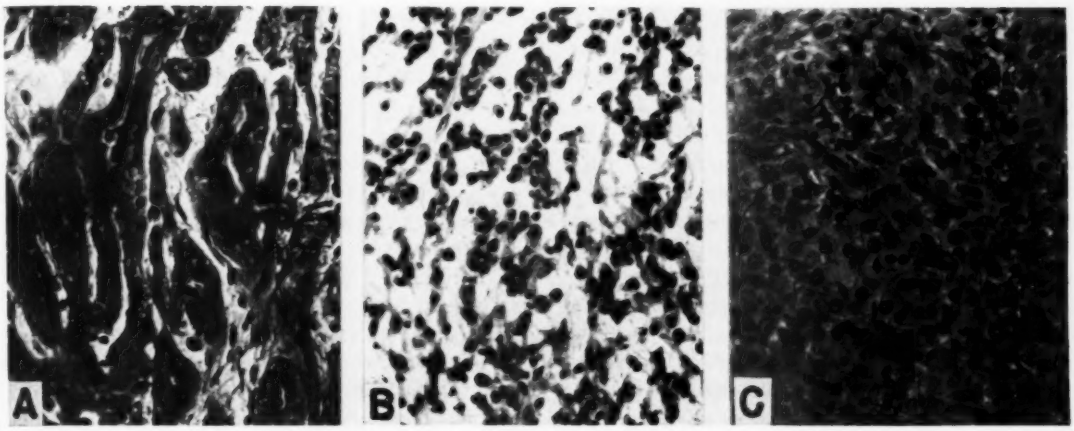

FIGURE 2.—Tumor 1643. *A*, Original adenocarcinomatous growth, with gland formation. *B*, Generation 4, 1 area shows considerable intercellular substance and many pyknotic nuclei; no suggestion of gland formation. *C*, Another area, resembling an anaplastic carcinoma with closely packed cells. Hematoxylin-eosin stain. × 300.

or absent. Some of the neoplastic cells blended with stroma, and single or small groups of cells were intimately surrounded by reticulum and collagen.

In later generations, this tumor seemed to become established in two distinct patterns, which often occurred together and were blended with each other. In one, the spindle-shaped cells were separated by an abundant intercellular substance (fig. 2, *B*); in the other the cells were closely packed, and the tumor resembled an anaplastic carcinoma (fig. 2, *C*). The cells were generally oval to spindle-shaped, they varied considerably in size, and many nuclei appeared to be pyknotic. Mitotic figures were infrequent. No cytoplasmic fibrils were made out with the phosphotungstic acid stain. The cytoplasm of many tumor cells contained granules of varying size, which stained red after treatment with periodic acid and Schiff's reagent, and these granules persisted after diastase digestion. Collagen and reticulum were abundant in the less cellular pattern, and the fibers were seen to be in close association with tumor cells. Mast cells were infrequent. Although the individual neoplastic cells were similar, the two forms of growth appeared unlike, and the apparent difference was probably due to the amount of intercellular substance and cell pyknosis. The blood vessels in both types were closely associated with tumor cells. The growth rate of this tumor through the eighth generation was similar in all generations and did not vary with the histologic appearance. Beginning with the ninth generation, when the tumor began spreading freely throughout the abdominal cavity and on the surface of the viscera, the growth rate became somewhat more rapid. Histologically, the tumor resembled the more cellular portions of the tumor described previously.

This tumor has been transformed during a series of transplantations from a distinctly epithelial structure to a type resembling sarcoma. It is difficult to diagnose such a tumor. This problem was first encountered with mammary tumors (*3*) and later with lung tumors (*4*). Opinions differ whether this change is due to a malignant transformation induced in the connective tissue stroma by the carcinoma cells, whether the original tumor was mixed, or whether it represents progressive anaplasia of the carcinoma cells, so that they lose all suggestion of a glandular arrangement and assume a spindle form. It is possible that any one of these explanations may sometimes be correct. The inconclusive evidence from the history and morphology of tumor 1643 makes it impossible to decide which of the foregoing explanations may account for the changes observed.

Biochemical Characteristics

The catheptic activity of tumors 2226 and 1643 was investigated by Maver, Dunn, and Greco (*5*), who reported that tumor 2226 had less systemic effect on the catheptic activity of the tissues studied than did tumor 1643. The catheptic activity of the primary first and third generations of tumor 1643 remained unchanged although there was morphologic evidence of alteration during this time.

Carcinoma 2226 shows the same high rate of glycolysis typical of tumor tissue, and is excellently adapted to glycolytic studies.[3] The respiration of carcinoma 2226 as well as other tumor tissue was shown by Burk et al. (*6*) to be inhibited with cobalt. This inhibition can be prevented by adding histidine before the cobalt damage has taken place.

Greenstein and Leuthardt (*7*) studied the enzymatic activity of aqueous extracts of tumor 2226, Jensen sarcoma, and hepatoma 31. The data showed that the arginase, catalase, dehydropeptidase I and II, and ribonucleodesaminase activities of tumor 2226 and Jensen sarcoma are similar.

SUMMARY

Two transplantable rat tumors were induced in Buffalo-strain rats by administration of N-acetyl-2-aminofluorene. Carcinoma 2226 arose within the orbital cavity, grew rapidly after transplantation, and metastasized to the lungs. The basic cell type and structure remained uniform; although the transplants varied considerably in the amount of stroma which separated the epithelial cells; mitotic figures were frequent.

Tumor 1643 arose in the subcutaneous area in the region of the groin and appeared to be of mammary-tissue origin. It grew less rapidly than did tumor 2226 and did not metastasize to the lungs. This tumor has become established in two distinct patterns which are often together. In one, the spindle-shaped cells are separated by abundant intercellular substance; and in the other, the cells are closely packed. Mitotic figures are infrequent. This tumor is another instance of morphologic transformation of a tumor during transplantation.

Some biochemical characteristics of these tumors are reviewed.

REFERENCES

(*1*) Wilson, R. H., De Eds, F., and Cox, A. J., Jr.: The toxicity and carcinogenic activity of 2-acetaminofluorene. Cancer Research 1: 595–608 (1941).

(*2*) McManus, J. F. A.: Histological demonstration of mucin after periodic acid. Nature, London 158: 202 (1946).

(*3*) Haaland, M.: Contributions to the study of the development of sarcoma under experimental conditions. 3d Scient. Rep., Imp. Cancer Research Fund, pp. 175–261 (1908).

(*4*) Andervont, H. B.: Pulmonary tumors in mice. Further study on the serial transmission of lung tumors occurring in inbred mice. Pub. Health Rep. 54: 1519–1524 (1939).

(*5*) Maver, M. E., Dunn, T. B., and Greco, A: The catheptic activities of the tumors and tissues of tumor-bearing rats. J. Nat. Cancer Inst. 9: 39–41 (1948).

(*6*) Burk, D., du Buy, H., Fischer, C., Hearon, J. Z., Hesselbach, M., Walter, J., Levy, H., Olson, B., Piggott, W. R., Schade, A. L., and Vinson, S.: Metabolic inhibitions of microorganisms and animal tissues and tumors by penicillin, penicillin impurity, streptomycin and cobalt and some applications to metabolic identification, assay, and mode of action. Rep. Antibiotics Study Sect., N. I. H. May 2, 1947. 24 pp.

(*7*) Greenstein, J. P., and Leuthardt, F. M.: Note on the enzymatic activity of normal and neoplastic tissues of the rat. J. Nat. Cancer Inst. 6: 317 (1946).

[3] Burk, D.: Personal communication.

THE GROWTH IN VITRO OF SINGLE ISOLATED TISSUE CELLS [1]

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INTRODUCTION

One obstacle to the more accurate use of tissue culture, particularly in cancer research, has been the inability to grow a single, isolated, tissue cell into a clone or pure culture. Even with those cultures which have grown in vitro for a period of years and which appear to be homogeneous, there has been no assurance of purity of cell strain, since all cultures have originated from the mixed population of cells in a tissue explant.

There have been many efforts to grow a single vertebrate tissue cell, with little success. Although Fischer (*1*), studying the Rous sarcoma cell, and Moen (*2*), working with the normal mononuclear blood cell, secured single-cell division, this growth occurred only in the presence of other tissue cells. Olivo (*3*), using embryonic heart and skin, demonstrated occasional single-cell division but found 26 cells to be the minimum number which proliferated in vitro to yield a colony.

Several hypotheses have been proposed to explain the failure of the isolated cell to live and reproduce. One of these, advanced by Fischer (*1,4*), is that the tissue cell has a limited autonomy as a physiological unit and that initiation of cell cleavage in a tissue depends on a system of internal exchange between cells through direct protoplasmic bridges or contacts; for proliferation of tissue cells, these cellular connections must be intact. This hypothesis would seem to preclude further experimentation in growing single, isolated cells.

A second hypothesis is that failure in growing single cells results from the technical difficulty in isolating a cell by microdissection without mechanical injury. In view of the careful isolation method without microdissection used by Fischer in his studies (*1*), this hypothesis as a complete explanation seems improbable.

A third hypothesis, suggested by the work of Earle and Thompson (*5*) and Earle [2] and supported by Moen (*2*), is that the single cell fails to grow because even our best culture media are so inadequate as to need extensive modification by the cells before utilization. While a large clump of tissue cells can adapt a reasonable volume of the usual culture medium, one cell grown in an identical volume is unable to adjust the fluid sufficiently for survival and proliferation.

On the basis of this concept, an attempt has been made (1) to reduce the amount of culture medium bathing the single cell to that volume which the cell can adjust and (2) to supply the cell with a culture medium already adjusted by the growth of large cultures of living cells. With this approach it has been possible to grow single cells of one strain completely isolated from other cells and to obtain from them cultures comparable in size to those derived from multicellular explants.

MATERIALS AND METHODS

Source of Cells

The cells of this study were from Earle's strain L (*6*), which originated from normal, subcutaneous, connective tissue of an adult C3H strain mouse. This strain of cells had been treated with 20-methylcholanthrene and had produced sarcomas on injection into C3H strain mice. The cultures were grown as described by Evans and Earle (*7*) under perforated cellophane in Carrel D 3.5 flasks. Cultures were used when a diffuse growth of isolated cells could be observed on the glass floor of the flask under the cellophane (fig. 1): cell isolations were made approximately 6 hours after a change of the fluid medium on the culture.

Isolation of Single Cells

The general arrangement of apparatus used in isolation of the single cell is shown in figure 2. Single cells were isolated free-hand under a dissecting microscope equipped with 10 × wide-field oculars and 4 × objective. Each cell was picked from the glass floor of the flask by means of a micropipette (fig. 3) [3], inserted through a perforation

[1] Received for publication August 16, 1948.

[2] EARLE, W. R.: Unpublished investigations.

[3] The micropipettes (fig. 3, *A*) were drawn from pyrex tubing 2.8 mm. outside diameter and 1.4 mm. inside diameter. The tubing was drawn to an internal diameter of approximately 0.1 mm. The total length of the micropipettes averaged about 125 mm. of which approximately 25 mm. at one end consisted of unconstricted tubing. The unconstricted portion was plugged at its open end with cotton. Just before use, the unconstricted

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FIGURE 1.—Nine-day old culture of strain L cells growing in a D 3.5 Carrel flask under perforated cellophane. This shows the type of culture from which single cell isolations were routinely made. × 200.

in the cellophane. Several individual cells were drawn into each pipette, an attempt being made to distribute them along its length.

During cell isolation the culture flask was gassed continuously with 5 percent carbon dioxide in air to control the pH of the culture fluid during the dissection. This gas mixture was first passed, at a pressure of about 8 pounds/square inch, through a gas humidifier (fig. 2).[4]

Each micropipette was flushed with chicken plasma directly before use. This adsorbed plasma formed a thin clot to which the cells were found to anchor more readily than to the glass of the wall. With the pipette the cells were either dissected or blown loose from the glass surface of the flask and were sucked into the pipette. A bubble of air was next introduced into the pipette after the suspension of single cells, and finally a small amount of plasma clot mixture, prepared by mixing one drop of fluid medium with one of chicken plasma. This mixture clotted slowly to seal the end of the micropipette.

The capillary tube was then broken from the base of the pipette. Each micropipette thus yielded a capillary tube approximately 100 mm. long, containing one or several isolated cells, with the distal end of the tube filled with plasma. The proximal end of this capillary was immediately sealed[5] under a dissecting microscope fitted with

end of each micropipette was attached to a cotton-packed tube (fig. 3, *B*) by means of a short piece of rubber hemocytometer tubing, and from this a piece of rubber tubing (fig. 3, *C*) led to a mouthpiece (fig. 3, *D*). This cotton-packed tube served as a bacterial air filter.

[4] This consisted of a gas dispersion tube with a medium porosity sintered glass disk (fig. 2, *B*) inserted into a liter Erlenmeyer flask of distilled water. After bubbling through the water, the gas was freed of water droplets by passing consecutively through glass beads, then cotton contained in a tube (fig. 2, *C*). The gas was then sterilized by filtering through a sterile (autoclaved) 10-ml. syringe barrel packed with cotton (fig. 2, *D*). The syringe was fitted with a 3½ inch, 18-gage platinum-iridium needle (fig. 2, *E*), which was flamed before each use. This needle was bent at such an angle as to jet the gas against the inner surface of the roof of the culture flask (fig. 2, *F*).

[5] To do this, under a second microscope, a platinum wire 0.21 mm. in diameter was mounted (fig. 4) and was heated electrically. On this wire a small bead of glass was fused from a fine pyrex rod. The capillary was then sealed by touching its open end to the molten glass bead.

During the local heating and subsequent cooling of the proxi-

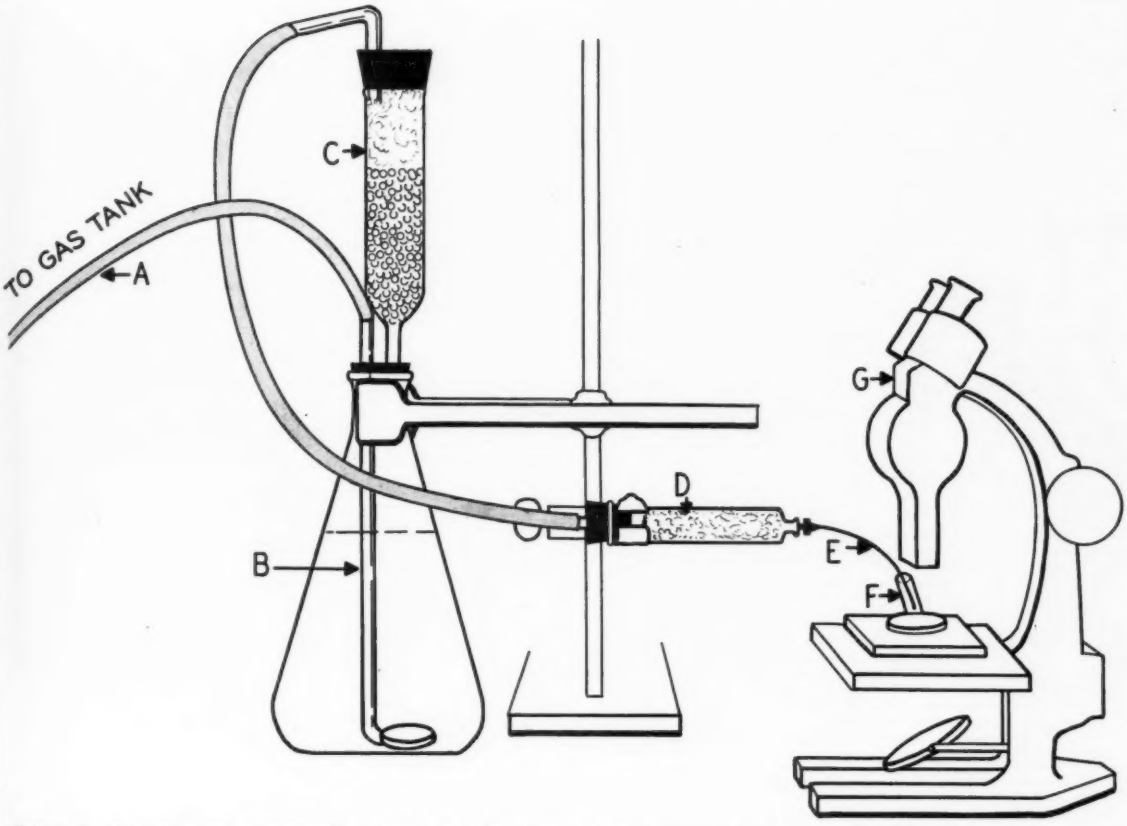

FIGURE 2.—Arrangement of apparatus used in cell isolation. *A*, tube from tank of 5 percent carbon dioxide in air; *B*, gas dispersion tube; *C*, tube of glass beads and cotton; *D*, syringe barrel packed with cotton to serve as bacterial filter; *E*, needle; *F*, Carrel flask containing culture, in flask holder; and *G*, dissecting microscope.

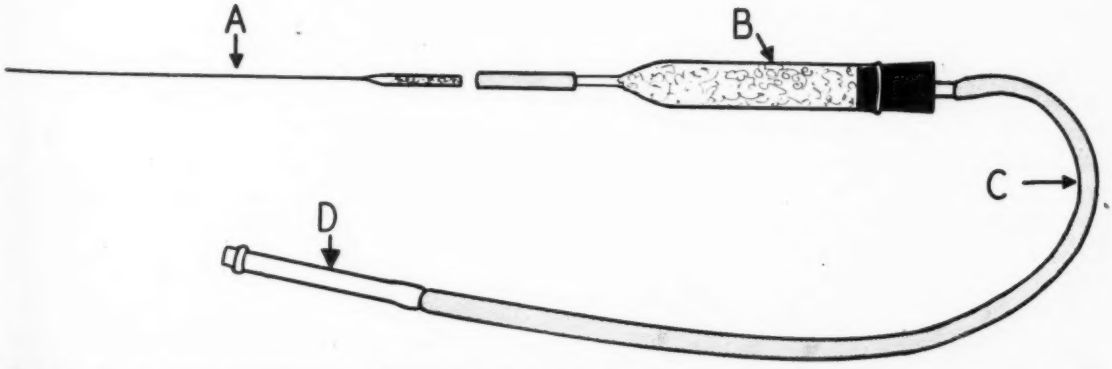

FIGURE 3.—Micropipette assemblage: *A*, micropipette; *B*, tube filled with cotton to serve as bacterial air filter; *C*, rubber tube; and *D*, mouthpiece

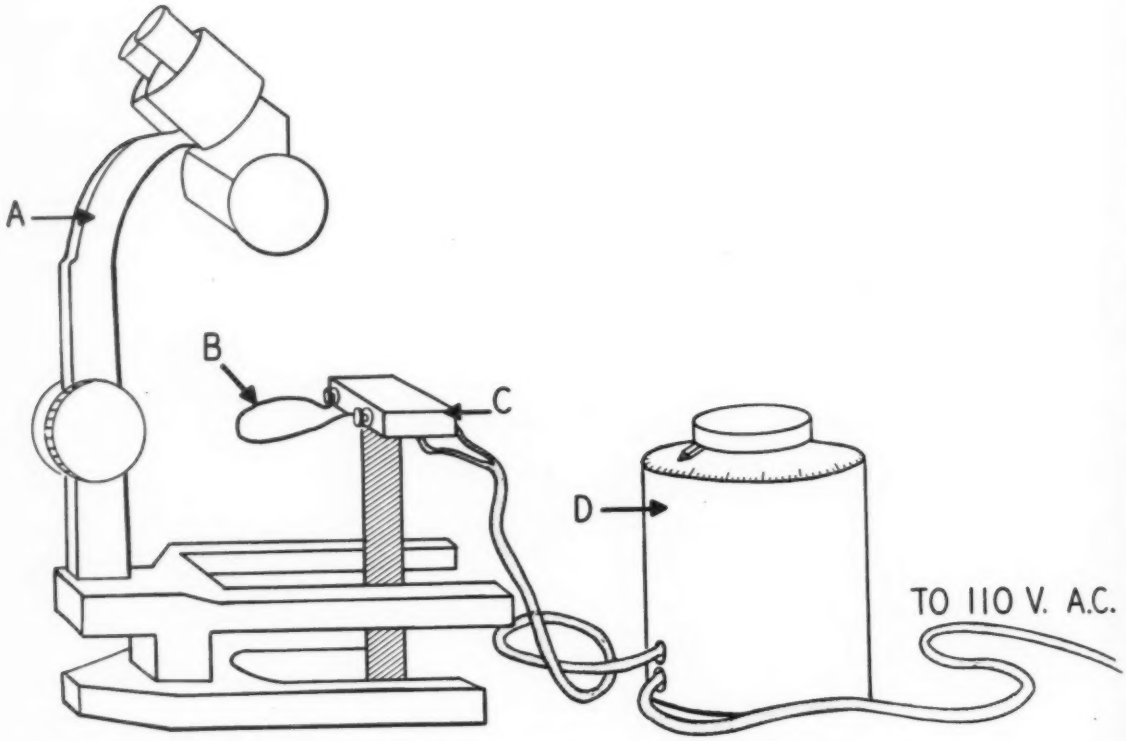

FIGURE 4.—Apparatus for sealing of capillary tubes. *A*, dissecting microscope; *B*, platinum wire; *C*, support for platinum wire; and *D*, variable transformer.

0.75 × objective and 10 × oculars (fig. 4), after which the distal end was finally sealed.

SELECTION OF CELLS FOR PLANTING

The sealed capillary tubes were incubated at 37.5° C. for 15 to 20 hours, then mounted in oil [6] on a slide, under a 24-x-75-mm. No. 1 cover slip. These preparations were examined microscopically with a 5.5-mm. objective and 10 × oculars, and well-isolated, healthy, single cells were selected. The distance of each selected cell from one end of the capillary tube was measured and recorded, and the tube was placed in a rack, oriented so that the end of the capillary from which measurements were made was toward the left. This orientation of the capillary made it possible to locate each selected cell from the recorded measurement.

PLANTING OF CAPILLARIES

The sealed capillaries were sterilized by immersion in chloroform for 15 minutes. With sterile forceps, each capillary tube was then transferred to a sterile 150-mm. petri dish. By means of a millimeter scale under the dish, the location of the selected cell was determined by measurement from the end of the capillary. A section of capillary 4 to 5 mm. long with the selected cell approximately midway of its length was then cut from the capillary by pressing on it with the blunt edge of a sterile Bard-Parker knife, blade No. 23. Each

mal end of the capillary, there was first expansion, then contraction of the air in the heated end. The slow clotting of the plasma at the distal end allowed time for adjustment of this pressure change. After the solid clot had formed at the distal end, the distal end was also sealed with a molten glass bead. In the final sealing of the distal end, the plasma plug prevented movement of the fluid and cells within the capillary. This maintenance of approximately atmospheric pressure in the air at both ends of the fluid column was essential in preventing fluid movement and consequent dislocation of the cells when small segments were later cut from the capillary.

[6] Shillaber's immersion oil, high viscosity, obtained from R. P. Cargille, 118 Liberty Street, New York City.

short capillary tube was grasped with sterile forceps [7] and inserted into a Carrel D 3.5 flask containing 0.5 ml. chicken plasma and 0.5 ml. culture fluid. The capillary tube was oriented in the middle of the flask. After the plasma in the flask solidified, an additional 1 ml. of culture fluid was added; the supernatant air in the culture was replaced by the gas mixture, by using the procedure described, and the flask was sealed with a rubber stopper. Every 48 hours the old fluid was removed, fresh fluid added, and the culture gassed and resealed.

Preparation of Fluid Culture Medium

Two types of fluid culture medium were used. The first, designated as normal fluid culture medium, was similar to that already described by Earle (*6*) and consisted of 40 percent horse serum, 20 percent chick embryo extract (1:1), and 40 percent saline.

The second type designated as conditioned medium, was prepared by growing large plasma cultures of strain L cells in the normal culture medium for 18 to 24 hours at 37.5° C. This fluid, which had been acted upon by the growing tissue, was then drawn off the cultures and fortified with a sterile glucose solution [8] to a concentration of 100 mg. glucose per 100 cc. of culture medium.

To eliminate extraneous cells, this conditioned medium was centrifuged in 10 x 100 mm. tubes at 2,500 r. p. m. for 15 minutes, during which process a sterile plug of absorbent cotton was thrown down through the fluid. The supernatant was then drawn off and frozen for about 30 minutes in dry ice. On microscopic examination there was no evidence of any extraneous cells in the culture fluid.

Methods of Observation

The condition and cleavage of individual single cells within the capillary were followed under a miscroscope, fitted with 10 × oculars and 5.5-mm. objective and mounted in a viewing box maintained at 37.5° C. Photomicrographs at 200 × were taken with the photographic equipment described by Earle (*8*) although the number of these was limited since the intensity of light used for photography appeared to injure the preparations.

RESULTS

With this procedure, about 60 sealed capillaries could be prepared in 3 hours with the assistance of a technician. Approximately 75 percent of these, after 24 hours of incubation, yielded healthy, well-isolated, single cells. An average of 2 single cells per capillary tube could be used for transfer to the Carrel flasks. In cutting the capillary and planting the short segment, about 40 percent of these cells were dislocated either due to slight fluid movement within the capillary at the time of cutting or to rapid emptying of the capillary segment on insertion through the surface film of fluid in the Carrel flask. Of the remaining 60 percent, about half survived the 24-hour period after transfer to the Carrel flask. During this time a slow diffusion of fresh culture fluid into the capillary was taking place. Only about 4 percent of the cells survived for 48 hours after planting and could be grown to clone cultures.

The growth curves of 4 single cells grown in conditioned medium in capillary tubes are presented in figure 5. Other curves showing the division rate of clumps of 2 to 10 cells are not presented since the initial latent period and the general slopes of growth curves are similar to those of the single cell cultures.

All growth curves show a latent period of 2 to 3 days from the time of cell isolation until the first cell division. Although much of this delay in cell growth was probably caused by the 4-hour to 6-hour chilling during the planting of the capillary segments, some of this lag may have represented the time for the cell to effect changes adjusting the culture medium.

From the second to the third day after isolation, the rate of cell cleavage varied from zero to three divisions per cell per 24-hour period. From the third to the fifth day the rate varied from five-tenths to three and five-tenths divisions per cell per 24-hour period. After this the rates tended to drop slightly. This change in growth rate does not mean, however, that the average mitotic activity of cells decreased over this period, for variable

[7] Dumont jewelers' forceps No. 3C obtainable from Greenan Watch Supply Co., Washington, D. C.

[8] Sterilized by filtration through an .03 Selas filter, obtainable from the Selas Corporation of America, Erie Avenue and D Street, Philadelphia 34.

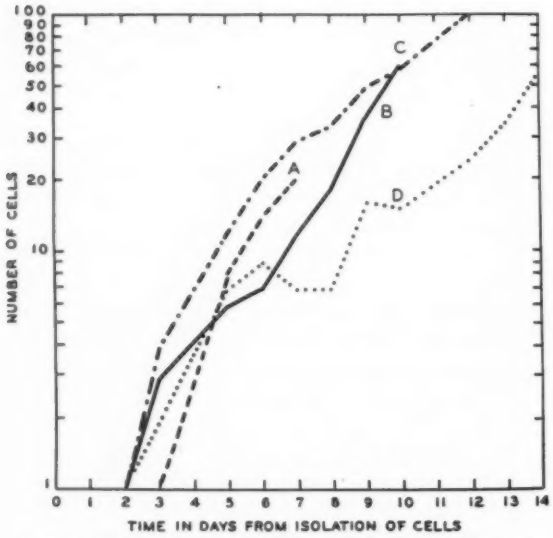

FIGURE 5.—Growth curves of four single cells. The number of live cells has been plotted on a semilogarithmic grid against time measured in days from the isolation of the single cells. Definitely necrotic, dead, or disintegrating cells have not been included in these counts. Culture A was closed due to bacterial contamination. Cultures B and C were grown from one source of conditioned fluid, and were closed soon after the 100-cell stage, due to an accident.

numbers of cells died. It was not practicable to secure accurate death rates with this type preparation because of cell migration and disintegration of dead cells.

Figure 6, *A*, shows 1 of these cells (fig. 5, *B*) in a capillary segment, embedded in conditioned medium plasma in a Carrel flask. The photograph was taken 2 days after isolation of the single cell and 22 hours after the capillary was planted in the Carrel flask. Figure 6, *B*, shows the same preparation 65 hours later when the cell had proliferated to yield 6 cells. Figure 6, *C*, is a photograph taken 48 hours later when there were 12 live cells and 1 dead cell present. A comparison of these 3 figures gives an idea of the degree of migrational activity of the cells.

The growth curve of one single-cell culture (fig. 5, *D*) reflects the death of a number of cells between the sixth and eighth day of growth. The cause of this injury was not clear. Figure 7 shows this culture 16 days after cell isolation when the first cells were observed to be emerging from one end of the capillary. Figure 8 is a photograph of one end of the capillary showing the healthy condition of the emerging cells. Figure 9 shows the growth of this culture 42 days after isolation. At this time, the culture was taken off conditioned medium and grown in the normal culture fluid. By the sixty-fifth day, this culture had covered the floor of the D 3.5 flask and was divided to produce four explants, which have since been subcultured and are growing luxuriantly under cellophane.

In all preparations, single cells located near the ends of the capillary segments either died before division or divided and the daughter cells died. The only single cells to divide were those located near the center of the capillary tube. Later, when the cell density increased, the center of the capillary tube appeared least favorable for cell growth, and the cells of this zone usually became necrotic and died, while the cells near the open ends of the capillary were still actively proliferating (fig. 10). It is possible that at this stage the dense cell growth at the open ends of the capillary blocked the diffusion of culture fluid to the center of the tube.

Although the number of trials was limited, no single cell divided when in normal medium, but cell clumps of 4 and 6 cells did proliferate to fill the capillary tube. Once the cells migrated through the open ends of the capillary tube into the plasma in the flask, it was necessary to use conditioned fluid to maintain them. Unless conditioned fluid was used, cell injury and necrosis appeared within a few days after the cells emerged. An instance of this is shown in figures 11, 12, and 13. Figure 11 shows cells which had emerged from the capillary tube and which exhibited a fairly healthy appearance even though grown in normal culture fluid. Figure 12 shows the same preparation 3 days later at which time there was obvious cell necrosis and degeneration. Figure 13 shows the same culture 41 days later, at which time practically all the cells outside the capillary were dead. During the 41-day period, cells continued to migrate into the plasma of the flask, but the cells outside the capillary were unable to survive and to establish the culture.

Figure 14 is a photograph of a culture grown from six cells in normal culture fluid. By the fourteenth day of growth cells had emerged from one end of the capillary tube, and by the seven-

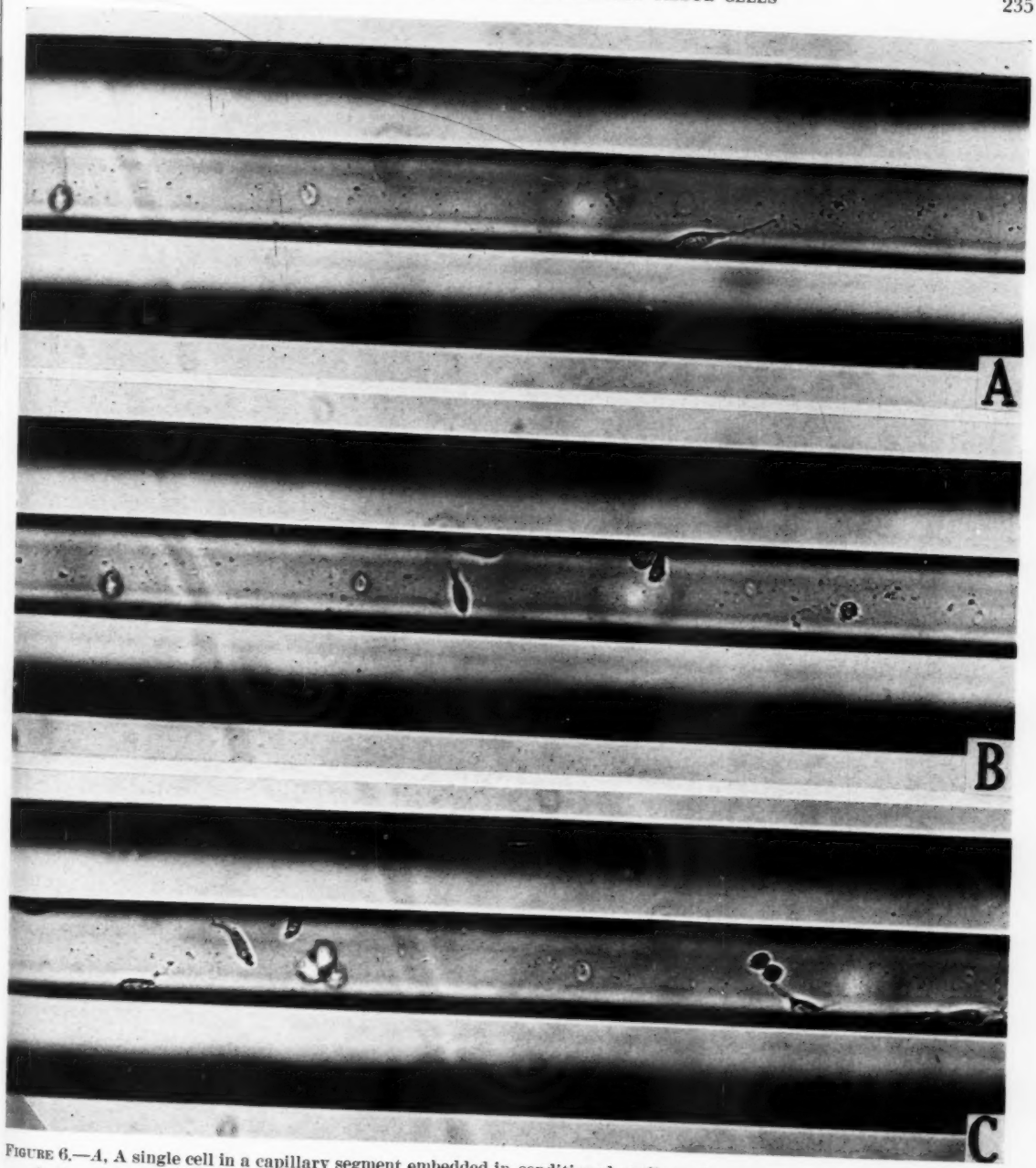

FIGURE 6.—*A*, A single cell in a capillary segment embedded in conditioned medium plasma on the floor of a Carrel D 3.5 flask. × 200. *B*, Same preparation as in *A* taken 65 hours later, 87 hours after planting, and 48 hours after a fluid change. The cell had proliferated to yield 6 cells, 1 of which cannot be seen due to curvature of the capillary. The cell at the right is dead. × 200. *C*, Same preparation as in *B* taken 48 hours later, 135 hours after planting, and 24 hours after the last fluid change, when there were 12 live cells and 1 dead. Several of the cells are out of focus. One cell was undergoing division. × 200.

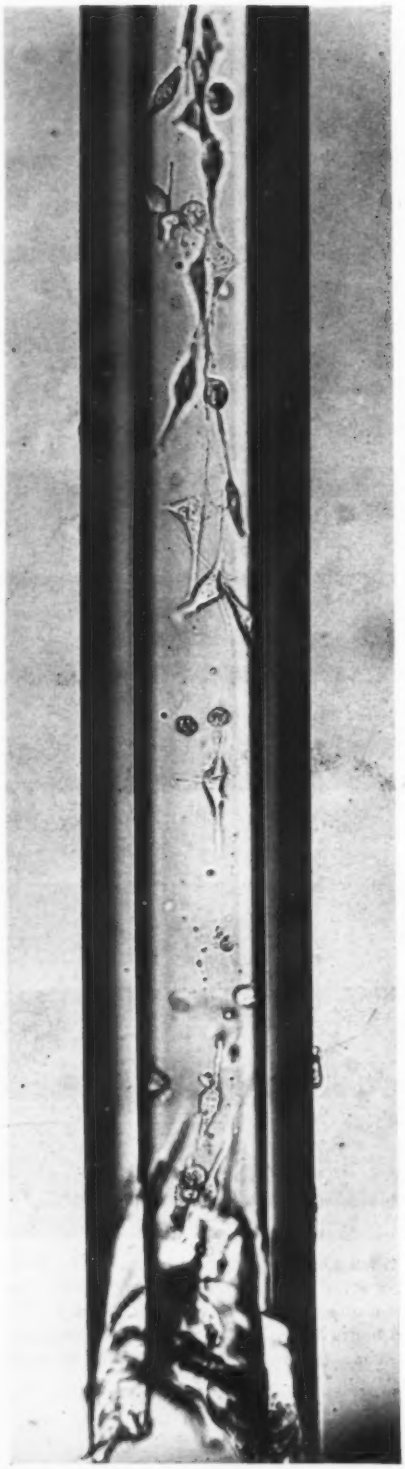

FIGURE 7.—Growth of single-cell culture in conditioned medium in a capillary segment, 16 days after cell isolation and 24 hours after the last fluid change. Note that the cells were beginning to migrate into the surrounding medium through the open end of the capillary. One cell can be clearly seen adherent to the outer surface of the capillary. × 200.

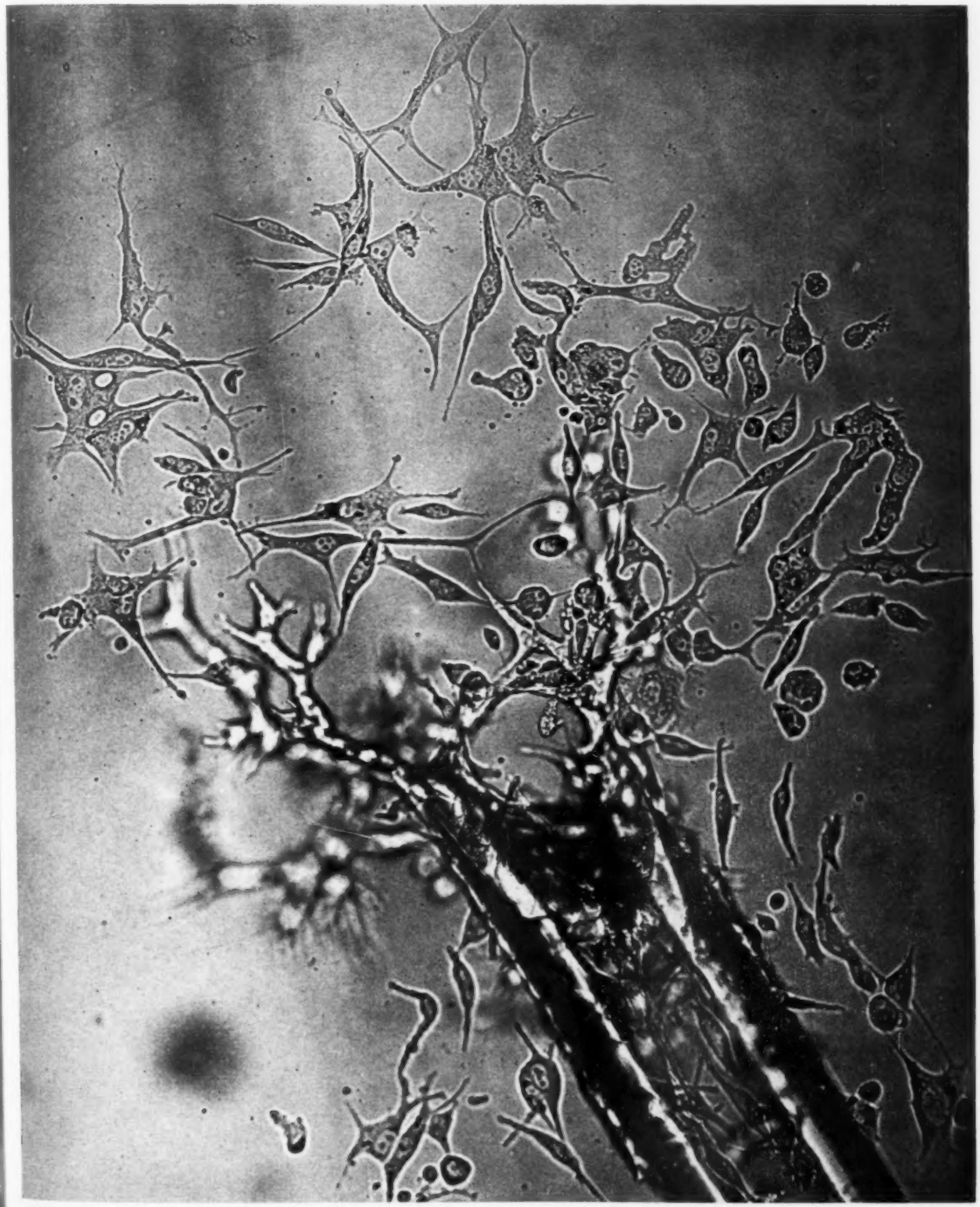

FIGURE 8.—The single cell culture shown in figure 7, photographed the twenty-eighth day of growth 24 hours after the last fluid change. The cells have migrated through the open end of the capillary into the plasma of the outer culture flask. Note the variation in cell size and shape. × 200.

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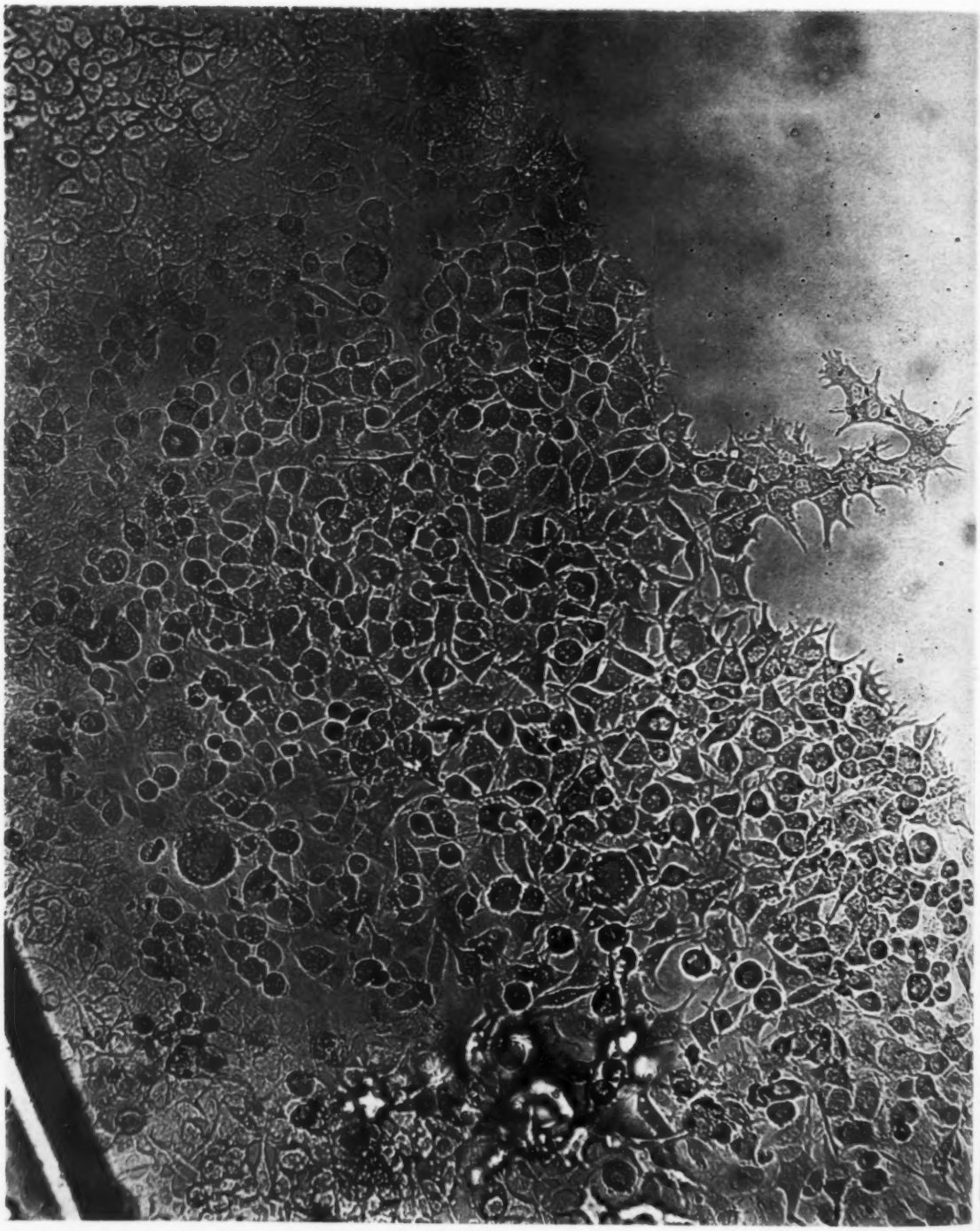

FIGURE 9.—Single-cell culture shown in figures 7 and 8, 42 days after isolation of single cell and 24 hours after the last fluid change. Note the cell morphology previously described as characteristic of strain L (*6*) and the occurrence of a number of giant cells. The capillary is shown at the lower left. × 200.

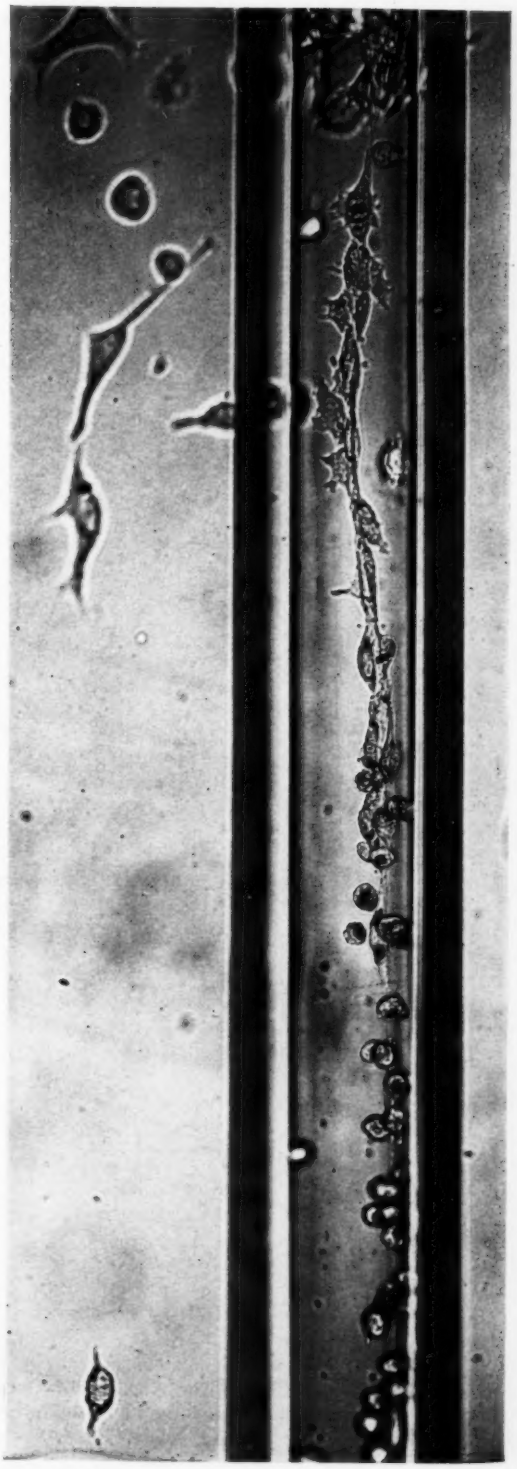

FIGURE 10.—Culture of cells growing in capillary segment showing healthy, actively proliferating cells at open end (top) and necrotic condition of cells near the central zone of the capillary segment (bottom). × 200.

FIGURE 11.—Cells grown in normal culture fluid migrating from the capillary tube into the outer culture flask. × 200.

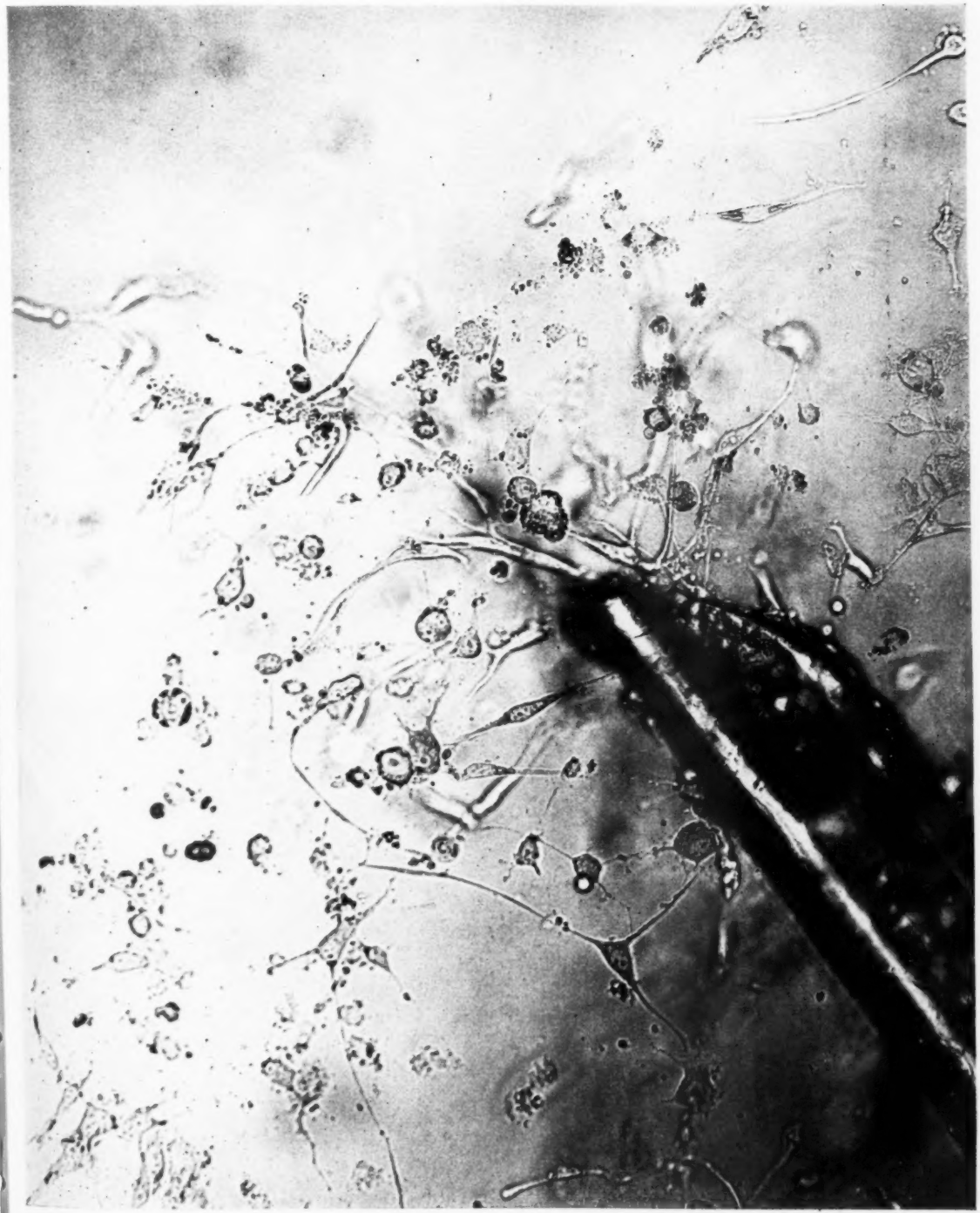

FIGURE 12.—Culture of figure 11, 3 days later, grown in normal culture fluid, showing necrosis of cells. × 200.

FIGURE 13.—Culture of figure 12, 41 days later, grown in normal culture fluid. Continuous migration of cells into the outer flask had occurred, but these cells had died. × 200.

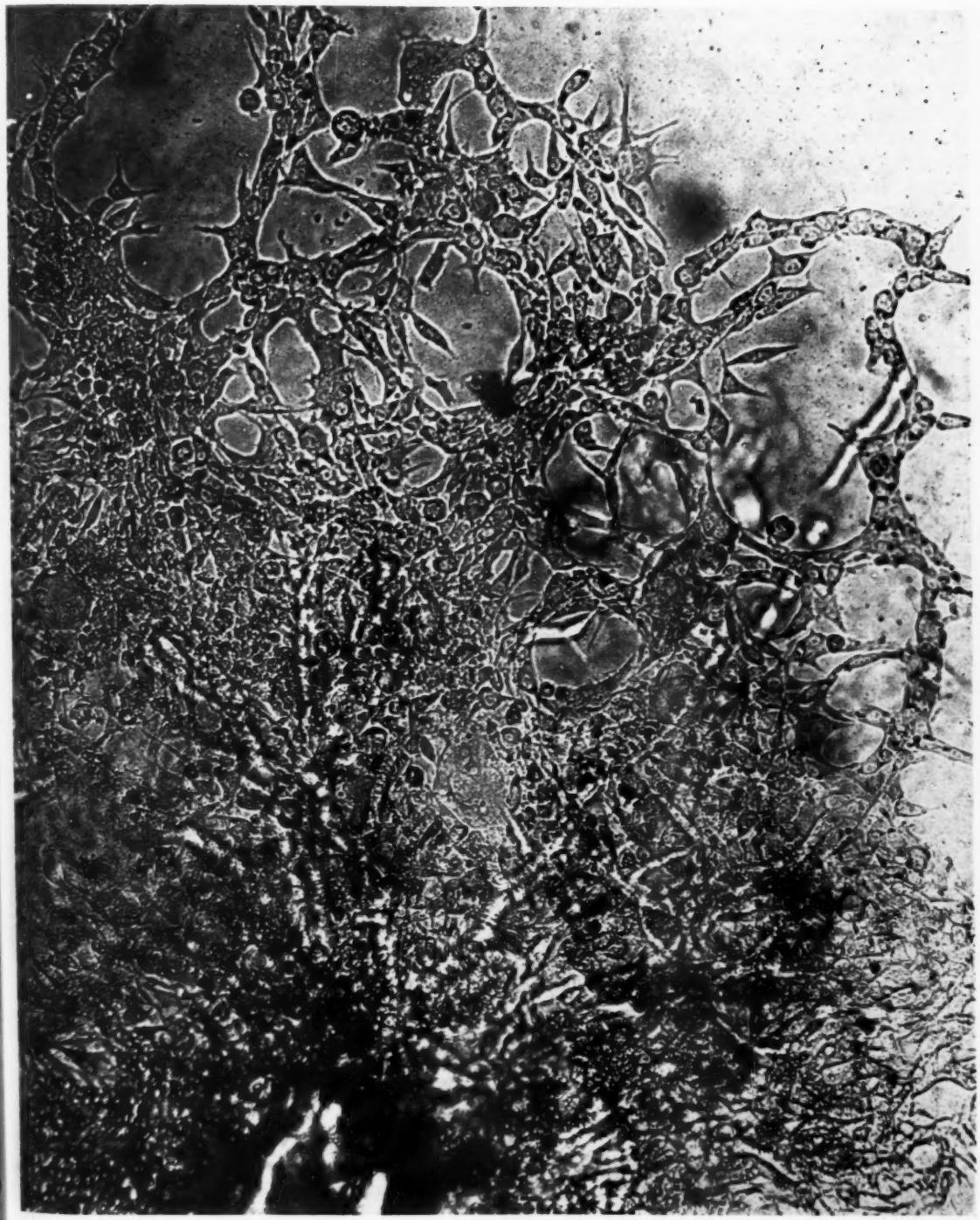

FIGURE 14.—Culture 6 days after 24-hour conditioned medium was added, 48 hours after the last fluid change. Note the healthy condition of the cells. × 200.

teenth day there was a healthy growth of cells in the plasma of the flask at each end of the capillary tube. By the nineteenth day, most of these cells had died. The cells within the capillary continued to divide, and successive waves of healthy cells migrated into the plasma of the flask. By the forty-first day, the majority of cells in the plasma outside the capillary tube were necrotic or dead, although numerous healthy cells were present in the capillary. At this time, conditioned medium was added, and 6 days later the photograph of figure 14 was taken. As may be seen, the conditioned medium had allowed the cells to grow into a healthy colony. At a later date, this colony was transferred to normal medium and covered the floor of the flask.

Although the number of experiments is limited, each of three cultures grown at different times on conditioned medium became established outside the capillary tube without visible sign of cell injury, whereas each of the nine cultures grown at different times on normal medium proliferated actively within the capillary but failed to survive and become established outside the capillary tube.

DISCUSSION

Since the primary approach to this problem lay in reducing the amount of culture medium to that volume which a single cell can adjust by its own metabolic activity, it was necessary to obtain some estimate of that volume. For an actively growing culture in which the cells are uniformly distributed over the floor of the flask, this volume was assumed to be the average volume of fluid bathing each cell, calculated as $\frac{\text{total volume of culture medium}}{\text{volume of cells in culture}}$. A culture of strain L cells, growing under cellophane from a strip explant 5 x 15 mm. in a Carrel D 3.5 flask for 30 days was estimated from measurement of oxygen consumption [9] to contain about 10 mg. wet weight of tissue, or, assuming a specific gravity of 1.0 for the cells, to have a volume of 10 mm^3. Since this volume of cells was bathed by about 1 ml. or 1,000 mm^3. of culture medium, each volume of tissue was bathed by 100 volumes of culture fluid.

The diameter of the rounded cell of cultures of this strain as measured from photomicrographs averaged 20μ. The cell volume was approximately 4,000μ^3. Each cell was thus required to adjust approximately 400,000μ^3 of culture fluid.

A capillary tube appeared to be the most practicable type of culture vessel which would theoretically allow the handling of such a limited volume of culture medium without evaporation, change of pH, or bacterial contamination. If the capillary, open at the ends, were immersed in a larger vessel of culture medium, it would be possible to effect a continuous slow change by diffusion of the culture medium around the cell; while if cell proliferation were obtained, the cells could migrate through the open ends of the capillary into the larger culture vessel.

By trial, it was found that a capillary of about 100μ internal diameter was the smallest bore tube experimentally practicable since those of smaller diameter were too easily clogged. The rate of diffusion of the medium into such a capillary was then estimated from tests in which capillaries were filled with physiological saline colored with dye, and the rate of diffusion of clear saline into them was measured. Uncolored saline was found to diffuse into the column of colored fluid in the capillary at 37.5° C. at a rate of about 5 mm. each 24 hours.

A capillary of 100μ-bore diameter containing 400,000μ^3 would have a length of about 51μ. This length was experimentally impractical, for diffusion into such a short capillary segment would change the culture fluid so rapidly that any alteration the cell introduced into the surrounding medium would be quickly dissipated. A longer capillary was therefore used although it was anticipated that with this longer tube, which did not reduce the volume of fluid to the calculated level, the adjustment of the culture medium by the cell would be incomplete. On actual trial this proved to be the case since in no instance did a single cell divide when isolated in a capillary in normal medium, although clumps of four or more cells were able to proliferate.

To adjust the culture medium further to the needs of the single cell, a culture medium conditioned by the growth of a large culture of cells was used. The conditioned culture fluid was drawn from cultures of at least 1 week's growth after the medium had been bathing the cells for

[9] BURK, D.: Personal communication.

18 to 24 hours, since at 24 hours after a fluid change cells appear to be most actively dividing. Glucose was added to this medium to insure replacement of glucose depleted during the conditioning of the medium (*9*) and to establish a glucose concentration between 1 and 2 gm. per liter. Whether a shorter or longer period of conditioning or further fortification of the culture medium would be advantageous has not yet been studied. In this conditioned medium single isolated cells were able to proliferate in the capillary, and the resultant cells were able to continue their proliferation when they migrated from the capillary into the medium of the flask.

No experiments have been carried out to determine if a single isolated cell can live in the conditioned culture medium outside the capillary; however, the importance of the reduced volume of fluid and restricted diffusion provided by the capillary tube is demonstrated by the fact that clumps of four or more cells which grew in normal culture medium within the capillary failed to survive and proliferate after migration into the larger volume of culture medium in the flask. The single cells or small groups of cells that survived and divided were always those located near the center of the capillary segment. Those located near its ends, where fluid altered by the cell tended to diffuse or wash away, did not survive.

The importance of the pre-conditioning of the culture medium in promoting cell growth is also demonstrated since single cells divided only in conditioned fluid. Although clumps of cells could be grown in normal fluid in the capillary, these cells failed to survive when they migrated into the normal medium outside the capillary. When conditioned medium was used, however, these outwandering cells were able to live and grow into a macroscopic culture.

The results of this study indicate that the culture medium mixture of horse serum and chick embryo extract, which is known to produce luxuriant growth of large cultures of strain L cells, is far from optimal for the growth of a single cell or of a few cells until the medium has been physically or chemically conditioned or altered by the cells. This conditioning of the medium appears to be more than a pH change. Although earlier attempts to grow small cell clumps at pH levels adjusted between 6.6 and 7.8 increased the survival time,[10] no cell proliferation occurred. The conditioning may involve one or more processes of detoxification, redox potential adjustment, or secretion of substances, such as growth-promoting agents or extracellular enzymes by the cells. While at the present time the nature of this change is unknown, its elucidation would augment our knowledge of the conditions necessary for the survival and growth of the living cell. In the study of these conditions capillary tube preparations have proved extremely sensitive indicators.

As yet this study has been limited to the mouse cell of strain L, which has produced sarcomas on injection. This strain was chosen because its macroscopic cultures grew well in the normal culture medium used. Whether other cell strains can be grown by these procedures must be determined. Since the basic experiments and reasoning[11] (*5*) which led to this method of attack were derived from in vitro studies of normal embryo chick heart, and since these same principles of restriction of the volume of culture medium and of pre-conditioning of the medium are known to be applicable in the culture of bacteria, protozoa, and lower coelenterates, there seems good reason to expect that this procedure can be used for other types of cells, the macroscopic cultures of which can be grown satisfactorily.

SUMMARY AND CONCLUSIONS

By means of procedures involving restriction of the volume of culture medium and pre-conditioning of this medium, culture conditions have been devised which with one cell strain have allowed the continued proliferation of single, completely isolated tissue cells.

A clone culture of strain L sarcoma-producing cells from the C3H strain mouse has been established.

Data obtained indicate that the culture media and culture conditions currently used are inadequate for the growth of single cells until the medium has been modified by the metabolic activity of living cells.

[10] [11] EARLE, W. R.: Unpublished investigations.

REFERENCES

(1) FISCHER, A.: Tissue culture. Levin & Munksgaard, Publishers, Copenhagen (1925).

(2) MOEN, J. K.: The development of pure cultures of fibroblasts from single, mononuclear cells. J. Exper. Med. 61: 247–260 (1935).

(3) OLIVO, O. M.: Potenzialita di accrescimento di poche cellule somatiche isolate. Arch. Ital. di anat. e di embriol. 30: 241–258 (1932).

(4) FISCHER, A.: Biology of tissue cells. G. E. Stechert Co., New York (1946).

(5) EARLE, W. R., and THOMPSON, J. W.: The influence of the size of the explant upon cultures of chick fibroblasts in vitro. Pub. Health Rep. 45: 2672–2698 (1931).

(6) EARLE, W. R.: Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in the living cells. J. Nat. Cancer Inst. 4: 165–212 (1943).

(7) EVANS, V. J., and EARLE, W. R.: The use of perforated cellophane for the growth of cells in tissue culture. J. Nat. Cancer Inst. 8: 103–119 (1947).

(8) EARLE, W. R.: Production of malignancy in vitro. II. Photomicrographic equipment. J. Nat. Cancer Inst. 4: 135–145 (1943).

(9) WILSON, H., JACKSON, E. B., and BRUES, A. M.: The metabolism of tissue cultures. I. Preliminary studies on chick embryo. J. Gen. Physiol. 25: 689–703 (1942).

REVIEW

SUNLIGHT AS A CAUSAL FACTOR IN CANCER OF THE SKIN OF MAN[1]

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The question whether sunlight is a major causal factor in human cutaneous cancer is not one to be answered by a single line of argument; it is the kind of question to which a satisfactory answer can only be given in terms of converging evidence along several lines and the lack of opposing evidence. The question itself is one of some practical importance, since, if the role of this agent can be established, it should be possible to design preventive measures. Seven years ago I reviewed the problem as it stood at that time and arrived at the conclusion that, while the then existing evidence strongly supported the idea that sunlight is a major cause of cancer of the skin of man, there were numerous places where the picture was not very clear (*1*). In the interim, further investigations have amplified and rendered more interpretable the earlier data. The newer studies strengthen the general concept at several points and at no place conflict with it, so that it seems possible, today, to take a much more definite position.

As I pointed out in my previous review, there are five principal lines of argument to support the conclusion that sunlight is a carcinogenic agent: (1) Cancer of the skin occurs most commonly on the hands and face (approximately 90 percent); (2) outdoor workers are the most prone to this type of cancer; (3) ultraviolet radiation of wavelengths present in sunlight induces cancer of the skin in mice and rats; (4) cutaneous cancer of man is more common in southern than in northern latitudes; and (5) persons who are relatively immune to sunburn, particularly Negroes, are also little susceptible to cutaneous cancer. A glance at this outline is sufficient to show that these arguments extend into several fields of knowledge including the clinical, experimental, epidemiological, and meteorological. Most of the progress in the last 7 years has been made in experimental studies on laboratory animals, and these have not only enhanced our knowledge of the mechanism of cancer but have permitted a more critical examination of the clinical, meteorological, and epidemiological evidence. In the present brief review, intended to bring the status of the problem up-to-date, the earlier evidence is not discussed except where necessary to show the bearing of later studies on the problem as a whole and only insofar as the problem of human cutaneous cancer is directly concerned.

CARCINOGENIC WAVELENGTHS

Interpretation of the evidence rests at many points on our knowing what wavelengths of ultraviolet radiation can produce cancer, and the solution of this problem depends upon experimental studies carried out with laboratory animals. For such animals this wavelength range for carcinogenesis has been determined with adequate accuracy to meet our general needs, but there appears to have been some recent misunderstanding in this regard, and it is necessary, therefore, to consider this phase of the problem at some length.

As stated in my earlier review (*1*), for all practical purposes the longest wavelength which induces tumors in experimental animals may be taken as approximately 3200A.[2] This was first clearly shown by the extensive studies of Funding, Henriques, and Rekling (*2*) who reported their work at the meeting of the Comité Internationale de la Lumière at Wiesbaden in 1938. Because their paper appears only in the final report of that Committee, not being included with those published in Strahlentherapie (*3*) as a preliminary

[1] Received for publication February 25, 1948.

[2] Experiment places this limit between the mercury lines 3130A and 3341A. The value 3200A is, therefore, chosen as a round number giving the approximate long wavelength limit of carcinogenesis.

report, it was not accessible to me at the time of writing my earlier review. I have since consulted their paper which describes extensive experiments with clear-cut results establishing the long wavelength limit at approximately 3200A. Roffo (*4*) had carried out earlier studies with rats which, as I showed in my review, could be interpreted to be in complete agreement with this general conclusion. In 1939 Rusch, Kline, and Baumann (*5*) performed experiments of the same kind as those of Funding, Henriques, and Rekling, of whose work they were excusably unaware, and arrived at the same conclusion. Subsequent experiments in our laboratory gave results in complete agreement with the above conclusions (*6*) and showed, in addition, that wavelengths at least as short as 2537A (*7*) can induce tumors in the skin of mice.

All these data are assembled in table 1 which shows that in no case have tumors of the skin of rats or mice been induced by ultraviolet radiation which did not include wavelengths shorter than 3200A. Particularly notable as indicating the ineffectiveness of all longer wavelengths are those experiments in which Roffo used sunlight as a source of ultraviolet radiation. Removal of the fraction of sunlight shorter than 3200A by means of window glass (transparent glass), which fraction is less than 0.2 percent of the sunlight of longer wavelengths, removed the carcinogenic activity.

Recently statements have been published, however, which assign carcinogenic action to longer wavelengths than 3200A (*8, 9*), a conclusion not justified by the evidence; and it is necessary to correct this impression since it has important bearing on more than one aspect of the present problem. The idea seems to stem from certain experiments carried out by Bain and Rusch with filtered mercury arc radiation (*10*); the wavelength limits of transmission by the filter having been taken, erroneously, as the limits of carcinogenic action.

The basis for the misinterpretation is best understood by reference to figure 1. The mercury arc, used as a source of ultraviolet radiation in these and most of the experiments listed in table 1, emits only certain wavelengths of radiation, and for practical purposes it may be regarded as being composed of a few discrete lines; these are represented in figure 1 by vertical lines, the height of the line indicating the relative intensity of the radiation at

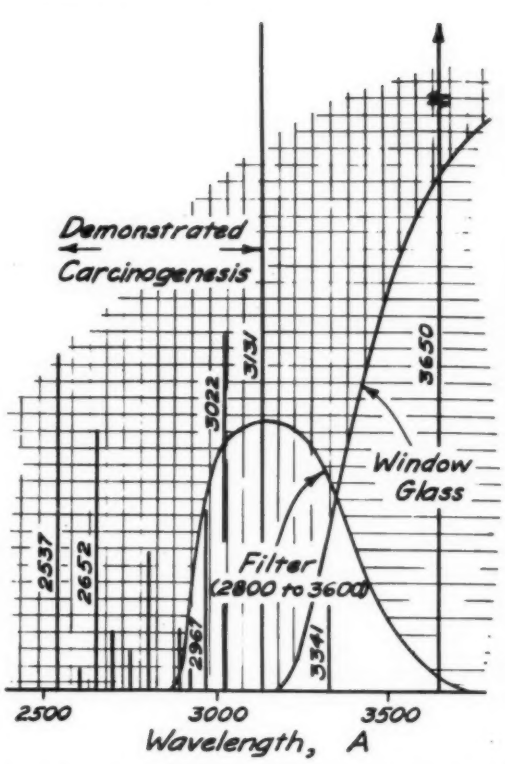

FIGURE 1.—The ordinates are arbitrarily chosen to show relative values only. The heavy vertical lines indicate the position and relative intensity of the lines emitted by the mercury arc (the intensities are for a type of atmospheric pressure arc (*11*)).

The light vertical hatching indicates the wavelengths removed by a filter of window glass. This glass is quite uniform in composition, but its exact transmission depends on the sample and the thickness. The curve is in good agreement with the spectral characteristics given by Funding, Henriques, and Rekling (*2*) and by Rusch, Kline, and Baumann (*5*) whose experiments most clearly establish the long wavelength limit for carcinogenesis as between the 3130A and 3347A mercury lines.

The light horizontal hatching indicates the wavelengths removed by the special filter used by Bain and Rusch (*10*). The curve for the transmission of this filter is drawn from an incomplete description and may not be strictly accurate, but it is sufficiently so for the descriptive purposes required herein.

the indicated wavelength. The relative intensities indicated are those measured in our laboratory for the particular type of mercury arc employed there (*11*). These intensities would have been somewhat different for the lamps used by the other in

vestigators, but the general relationships must have been similar and the wavelengths identical.

Table 1 shows that in no case did any of the several investigators find carcinogenic activity when a filter of window glass was introduced between the source of radiation and the animal. The transmission of a sheet of common window glass, is shown in figure 1; it transmits only wavelengths longer than about 3200A. The vertical shading indicates that part of the radiation removed by the window glass; that is, the mercury line 3131A and all shorter lines are removed, but the relatively weak 3341A line and the very strong 3650A line are passed, as well as numerous other strong lines of longer wavelength lying in the visible region of the spectrum which are not shown in the diagram. As reference to figure 1 shows, the radiation passing through window glass is not carcinogenic, so it may be assumed that the 3341A and all lines of longer wavelength have no carcinogenic action or so little as to be negligible. Reference to the table also shows that filters which pass the 3131A line and lines of shorter wavelength permit carcinogenesis by the mercury arc.

There is another type of mercury arc operating at very low pressure in which almost all the radiation is emitted in the 2537A line, resulting in practically monochromatic radiation. Experiments with such an arc have shown that it can produce tumors, although it is not very effective in doing so (*7*). Hence, wavelengths at least as short as 2537A have carcinogenic action in the mouse.

The approximate transmission of the filter used by Bain and Rusch (*10*) is also shown in figure 1, the radiation removed by it being indicated by horizontal shading. This filter cuts out virtually all radiation outside the range 2800A to 3600A. It thus allows to pass the important carcinogenic lines 2967A, 3022A, 3131A; but in addition the 3341A line and to a slight extent the 3650A line are passed, which have been shown by various experiments listed in table 1, including those of Rusch, Kline, and Baumann (*5*), to have no carcinogenic action. The finding that tumors were produced by mercury-arc radiation passing through Bain and Rusch's filter shows nothing new regarding the range of carcinogenic wavelengths. Their filter cuts out some of the lines of the arc that are known to be carcinogenic (e. g., 2537A) and permits the transmission of two which do not have carcinogenic action. Thus, estimates of carcinogenic effectiveness, based on the total

TABLE 1.—*Experiments determining the carcinogenic wavelengths*

Source	Investigators	Year	Animal	Filter	Shortest wavelength reaching skin in appreciable amount	Tumor induction
Mercury arc [1] in quartz (intermediate pressure).	Roffo (*4*)	1934	Rat	None	[2] 2302A	+
			do	Window glass [3]	[2] 3341A	−
	Funding, Henriques and Rekling (*2*)	1936	Mouse	None	[2] 2302A	+
				S&G BG3	[4] 2820A	+
				Window glass	[4] 3341A	−
				S&G BG8	[4] 4108A	−
	Rusch, Kline and Baumann (*5*)	1941	do	None	[2] 2302A	+
				Corning 970	[4] 2925A	+
				Window glass	[4] 3341A	−
				Corning 352	[4] 4046A	−
	Blum (*6*)	1943	do	None	2302A	+
				Pyrex glass	2967A	+
				Corex D	2699A	+
				Window glass [3]	3341A	−
	Bain and Rusch (*10*)	1943	do	Special	[4] 2925A	+
Mercury arc [5] (low pressure)	Rusch, Kline and Baumann (*5*)	1941	do	None	[6] 2537A	−
	Blum and Lippincott (*7*)	1942	do	do	2537A	+
Sunlight	Roffo (*4*)	1934	Rat	do	[2] 2900A	+
			do	Window glass [3]	[2] 3200A	−
Tungsten filament	Findlay (*37*)	1930	do	None	[2] 4000A	−
	Roffo (*4*)	1934	do	do	[2] 4000A	−
	Rusch, Kline and Baumann (*5*)	1941	Mouse	do	[2] 4000A	−
SI [7]	Beard, Boggess, and Von Hamm (*38*)	1936	Rat		[2] 2699A	+

[1] The emission of such lamps is similar to that illustrated in figure 1. Numerous other papers demonstrating carcinogenesis by such arcs are not included as they provide no further evidence regarding wavelength dependence.
[2] Based on my estimate. The characteristics of the source as well as the transmission of the filter have been considered in making these estimates.
[3] "Verre transparent," which I have assumed to be ordinary window glass, prevented carcinogenesis as did also several colored glasses which should have longer wavelength cut-offs.
[4] Based on author's statement. The characteristics of the source as well as the transmission of the filter have been considered in making these estimates.
[5] Unpublished result.
[6] Nearly all of the radiation emitted within the carcinogenic range is in the 2537A line.
[7] The dosage used was apparently inadequate (compare (*5*) and (*7*)).
[8] This is a combination of a tungsten filament and a mercury arc in special glass.

radiation transmitted by the filter, have little validity. There is no justification for assigning the approximate spectral limits of the filter as the spectral limits for carcinogenic action, which these investigators appear to do and others have accepted (*8*, *9*).

Actually, then, the status of the problem with respect to the spectral limits of the wavelengths effective in inducing cancer experimentally has not changed since my earlier review. These wavelengths, approximately 3200A and shorter, are referred to for convenience in the following discussion as to the carcinogenic radiation. Reference to figure 2 will orient the reader with regard to the relationship of this radiation to sunlight as a whole and to epidermal transmission.

Establishment of the long wavelength limit brings the induction of cancer into relationship with other biological effects of ultraviolet radiation, which have been studied in many different types of living systems, the killing of microorganisms and the production of mutations, among others. Insofar as studied, all these effects are limited to virtually the same wavelength range; that is, they are not produced by wavelengths longer than approximately 3200A.[3] The evidence cannot be gone into at length herein, but it is probable that such destructive effects are produced by the action of ultraviolet radiation on proteins and/or nucleic acids, the long wavelength limits for absorption by both nucleic acids and unconjugated proteins being about 3200A.

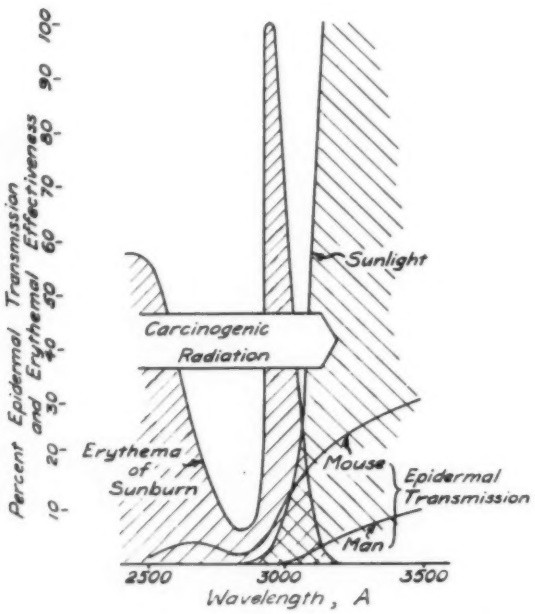

FIGURE 2.—Spectral relationships between carcinogenesis, erythema of sunburn, sunlight, and epidermal transmission. No ordinates are given for sunlight, the curve only indicating its lower wavelength limit and relative spectral intensity. The curves for epidermal transmission are from Kirby-Smith, Blum, and Grady (*12*). Note that sunlight includes only a small part of the wavelengths active in carcinogenesis and in erythema production.

Among the destructive effects of ultraviolet radiation is sunburn of human skin, and the relationship between this process and carcinogenesis needs consideration. A roughly quantitative description of the effectiveness of various wavelengths of ultraviolet radiation is given by the "erythema spectrum," which is reproduced in figure 2; that is, the curve there reproduced indicates the effectiveness of the various wavelengths in producing a just perceptible reddening of the skin of the area exposed. Such a curve, known as an action spectrum, must be interpreted with caution (*13*). The erythema spectrum in particular is not a strictly reliable measure of the sunburn mechanism (*14*), but it gives a rough index which is all that is needed for present purposes. There is a sharp minimum in this curve at about 2800A, undoubtedly resulting from the filtering action of the corneum, which acts as a nonparticipating layer superficial to the locus of action of the ultraviolet radiation in the living epidermal cells of the malpighian layer. The transmission of ultraviolet radiation by the corneum displays a minimum at 2800A, the spectrum resembling in shape that shown for mouse epidermis in figure 2. This minimum at 2800A corresponds to a maximum of absorption by typical proteins at this wavelength, and it is the absorption of ultraviolet radiation by these substances in the corneum which no doubt causes the minimum in the erythema spectrum.

[3] Effects of longer wavelengths in the ultraviolet have been described, but the energy required to produce them is much greater, and such effects probably have little relationship to the present problem since we have no evidence that they play a part in carcinogenesis.

It would be of interest for various reasons to know the shape of the action spectrum for sunburn at the level of the viable layers of the skin, and to compare this with the corresponding action spectrum for carcinogenesis and those for other biological effects of ultraviolet radiation. However, as I pointed out elsewhere, it is infeasible to obtain accurate measurements at this level for either sunburn in man (*14*) or carcinogenesis in the mouse (*6*), because of the complex optics of such systems.

To summarize, while the exact wavelength dependence of carcinogenesis, that is, its action spectrum, is not accurately known, its long wavelength limit is established with sufficient accuracy to make it clear that the carcinogenic wavelengths are approximately the same as those which produce destructive effects in living systems in general, and specifically sunburn of human skin (*15*). There is no evidence that the mechanism involved is not the same in all these processes, and it is reasonable to assume that carcinogenesis results from the same kind of primary photochemical reaction until good evidence can be brought forward to the contrary.

One other possibility needs mention. Precursors of vitamin D and other steroid compounds absorb in the same wavelength region as proteins and nucleic acid, and photochemical reactions involving these compounds are not completely ruled out as a basis for carcinogenesis. Strong evidence has accumulated, however, against this possibility (*16, 17*), but, since the arguments to be presented hereinafter do not hinge upon this particular point, it is not discussed further.

TYPES OF TUMORS

The distribution of tumor types found in human skin differs from that for tumors induced in experimental animals by ultraviolet radiation. This has been interpreted by some as indicating a lack of close relationship between these two phenomena. Upon examination in the light of recent studies, the discrepancy turns out to be more apparent than real, being largely interpretable in terms of the difference in depth of penetration of ultraviolet radiation into the two types of skin.

In man, cancers of the skin are almost all epidermal in origin, principally basal-cell carcinoma and squamous carcinoma, and there was considerable hesitancy on the part of some earlier investigators in accepting the fact that sarcomas are produced in mice and rats by ultraviolet radiation although both Roffo (*4*) and Funding, Henriques, and Rekling (*2*) reported them in large proportion. (See (*18*) for a discussion of the earlier findings.)

Since the appearance of my previous review, a long series of tumors induced in albino mice was examined histopathologically in this laboratory by Dr. Hugh Grady, and his diagnoses were confirmed by other pathologists of the National Cancer Institute (*19*). The results are summarized in table 2.

TABLE 2.—*Types of tumor induced with ultraviolet radiation in the ears of 440 albino mice (strain A) (data from Grady, Blum, and Kirby-Smith (19))*

Type of tumor	Depth of origin	Number of tumors	Percentage of animals in which found
Epidermal carcinoma	Epidermal	112	24
Spindle-cell sarcoma [1]	Sub-epidermal	440	95
Hemangio-epithelioma	do	5	1
Osteochondrosarcoma	do	1	.2
Sebaceous carcinoma	do	1	.2

[1] Most of these were of connective-tissue origin, but some probably were myosarcomas.

The great majority of tumors were of subepidermal origin, predominantly sarcomas, 96 percent of the animals had such tumors, while only 24 percent had epidermal tumors. The distribution of cutaneous tumor types induced in the albino mouse with ultraviolet radiation differs widely from that found in human skin.

In seeking an explanation of this discrepancy, it was at once suspected that the difference might be due to deeper penetration of the ultraviolet radiation into mouse skin than into human skin. There were no adequate comparative data for deciding this point, so Dr. Kirby-Smith made measurements of the transmission of ultraviolet radiation on separated epithelium both of the mouse and of man, by means of a special spectroscopic method designed to minimize the effect of scattering, which tends to decrease the apparent transmission of the epidermis (*12*). These measurements showed that wavelengths shorter than 3200A were transmitted by the epidermis to a much greater extent by mouse skin than by human skin, thus confirming our expectations. Two of the curves obtained are reproduced in figure 2. The skin from which the partic-

ular samples of epidermis were taken had undergone repeated exposure to ultraviolet radiation, and hence the epidermis had thickened as a result of hyperplasia so that the penetration was less than when the skin had not been so exposed.

The curves show that whereas in man virtually all the carcinogenic radiation is absorbed in the epidermis, a considerable amount passes through the epidermis of the mouse to reach the tissues of the corium. Let us examine the relationships carefully. In the case of either man or mouse, a large part of the carcinogenic radiation incident at the surface of the skin is absorbed in the nonviable corneum where it is lost so far as carcinogenic action is concerned. The fraction of radiation absorbed by the corneum varies with wavelength so that it is difficult to make a general estimate, but it is probably safe to say that within the carcinogenic range only about 10 percent of the radiation of any wavelength gets through to the viable tissue in the malpighian layer of the epidermis of man (*15*) but that a considerably greater amount penetrates to the corresponding layer in the mouse. In man, virtually all of this radiation which does pass through corneum is absorbed within the epidermis [4] so that carcinogenic action in man should be restricted virtually to the viable layer of the epidermis, since the carcinogenic radiation does not reach the deeper subepidermal tissues. The most prevalent cancers of human skin, that is, squamous-cell and basal-cell carcinomas, arise in the epidermis.

In the mouse, the situation is quite different. The viable epidermis of the malpighian layer is thinner than in man; and although a larger proportion of the incident radiation reaches this layer, only a small fraction is absorbed there. The remainder penetrates to the corium, and hence it is not surprising to find that many tumors of subepidermal tissues are produced. The small fraction of tumors of epidermal origin is consistent with the small fraction of carcinogenic radiation absorbed in the viable epidermis.

[4] Direct measurement indicates that a slight amount of radiation of the longest wavelengths that produce sunburn, say, 3000 to 3200A, penetrates below the epidermis of untanned human skin, and indirect physiological arguments support this, since inhibition of erythema is produced by large doses of these wavelengths, apparently by direct action on the vessels in the papillary layer of the corium (*20*). However, where human skin has been repeatedly exposed and has developed a much thickened horny layer, penetration below the epidermis is probably nil.

It is probable, of course, that different tissues have different susceptibilities to tumor induction, and this is indicated by the finding that the ratio of carcinomas to sarcomas changes with the interval between doses of ultraviolet radiation (*19*). It seems safe to conclude, however, that the apparent discrepancy between the findings in human skin and in albino mouse skin is to be accounted for largely by differences in the optical system encountered, so that one finds here no reason for believing that cutaneous cancers of man are not caused by the same agent that induces experimental tumors in the mouse, namely, ultraviolet radiation of wavelengths shorter than 3200A.

TOPOGRAPHICAL DISTRIBUTION

In my earlier paper I discussed at some length the suggestions that had been made with regard to topographical distribution of cancer of the skin and pointed out that no important generalization could be drawn other than that most cancers of the skin occur on the exposed areas, a high percentage (90 percent or more) appearing on the face. An interesting study of the topographical distribution of malignant melanomas recently appeared (*21*) which shows that this type of tumor is distributed with no particular reference to the face but rather closely parallel to the distribution of pigmented nevi. Thus, although a good many occur on the face, many are at other points seldom exposed to sunlight, particularly on the genitals and on the feet. Hence, sunlight probably plays little part in the development of these highly malignant tumors, which may in some instances originate in subepidermal regions which this agent does not reach. This does not mean that sunburn may not at times play a part as an irritating agent in the development of melanomas of exposed areas but that sunlight is certainly not a major factor in the induction of these tumors.

The high proportion of basal-cell and squamous-cell cancers on the face and other exposed parts of the body, plus much other evidence, serves to indict sunlight as the major factor in the induction of such tumors. The occurrence of an occasional squamous- or basal-cell cancer on an unexposed area of the body does not negate this conclusion, since such tumors are no doubt caused

at times by other agents.[5] On the other hand, the much more malignant, although fortunately much more rare, melanomas cannot be attributed in any major degree to the action of this agent, since they commonly occur on the unexposed parts of the skin.

TEMPORAL RELATIONSHIPS

In man most tumors of the skin appear after the fortieth year, although tumors appearing considerably earlier than this are not exceptional, and at most only about 3 percent of the white population of all ages develops such tumors. On the other hand, with appropriate dosage 100 percent of albino mice develop tumors in as little as 250 days (*22*). This discrepancy is sometimes presented as an argument against the application of the experimental evidence to the case of man. One deals here, of course, with that vague factor, biological time, and one is at a loss for a suitable relative measure for mouse and man, as is true in all questions of comparative incidence of cancer in different species no matter what the inducing agent.

The experimental studies indicate certain temporal relationships which permit the discrepancy to be partially rationalized. Difference in the quantity of carcinogenic radiation received from natural sunlight and that used in the experiments is an obvious factor to be considered. Any comparison is uncertain because of complicating physical factors; but as was pointed out by Kirby-Smith, Blum, and Grady (*12*), there is no reason to believe that any great discrepancy is involved, and the experiments of Roffo, in which he induced cancers of the skin of rats by exposing them to sunlight, supports this position.

Another possible factor is the interval between exposures. In strain A mice, it was found that a single exposure to ultraviolet radiation, no matter how severe, never resulted in the appearance of a tumor within the lifetime of the mouse. In fact, tumors could not be induced without repeated exposures covering a considerable time, and their development was accelerated by continuing the exposures almost up to the time of appearance (*23*). A great difference in times of tumor appearance was found, depending upon the interval between exposures: the less frequent the exposures, the greater the delay in appearance (*22*, *24*, *25*). Periods of respite from exposure to ultraviolet radiation were found to delay markedly the appearance of tumors (*23*, *26*).

If it is justifiable to extrapolate from the mouse to man, it may be argued from the above findings that a single severe exposure or even several repeated severe exposures are not likely to induce cancer of human skin, and this seems to agree with epidemiological findings. Cancer of the skin seems to occur most frequently among those persons who are exposed regularly to the action of sunlight; for example, it is higher in the Army and Navy of the United States (see (*1*)) than in the general population, and there is alleged to be a high incidence among cowboys in our Southwest, although a good basis of comparison seems lacking in the latter case. On the basis of this general evidence, it is not unreasonable to believe that the person who is severely sunburned for a short period during the summer, say at the seashore, is in much less danger of developing cancer than the person who is exposed continually throughout the year even though the immediate gross changes in the skin are less obvious.

DISTRIBUTION WITH LATITUDE

Statements have appeared from time to time to the effect that the incidence of cutaneous cancer is greater in those regions of the earth that receive the greatest amount of sunlight, but it has not been possible to make a critical analysis because of lack of quantitative data. Recent studies by Dorn (*27*), however, may be interpreted to indicate a relationship between latitude and incidence of cutaneous cancer and to provide a basis for such an analysis. This investigator carefully examined the incidence of various types of cancer in three groups of urban areas in the United States: (1) Chicago, Detroit, Pittsburgh, and Philadelphia; (2) San Francisco and Alameda County, Calif., and Denver; (3) Birmingham, Atlanta, New Orleans, Dallas, and Fort Worth. He designates these three groups, for his purposes, as "northern," "western," and "southern," respectively; but actually they form a series of nonoverlapping groups with respect to latitude, their mean latitudes,

[5] Kangri cancer and the cancers caused by carcinogenic chemical agents, e. g., the cancer of chimney sweeps and of workers in crude petroleum, may be mentioned in this regard.

weighted according to the populations of the various areas, being 40°, 38°, and 32°, respectively.

In figure 3, Dorn's incidence data for male and female white populations are plotted against latitude. A clear-cut north-south distribution of cutaneous cancer incidence is indicated for both sexes, being greater in the south than in the north. In these data the category, cancer of the buccal cavity, includes some tumors of the exposed part of the lip, which may account for the distinct north-south distribution of the incidence of such tumors. Cancer of all other sites does not show this relationship to latitude; actually, a small increase with increasing latitude in males is opposed by a small decrease in females. These statistics seem to indicate quite convincingly that some factor which varies with latitude markedly affects the incidence of cancer of the exposed parts of the body. We may examine the possibility that this factor is sunlight. To do so, it is necessary to understand rather clearly the extent of variation with latitude of the particular component of sunlight that is accused of inducing cancer. Total annual sunlight does not vary with latitude to nearly so great an extent as would be required to explain the differences in cutaneous cancer incidence, but this does not in any way deny the existence of a correlation between insolation and cutaneous cancer. The carcinogenic wavelengths, i. e., those shorter than 3200A (as established by animal experiments), comprise only a tiny fraction of total sunlight, less than 0.2 percent. This fraction changes greatly with latitude.

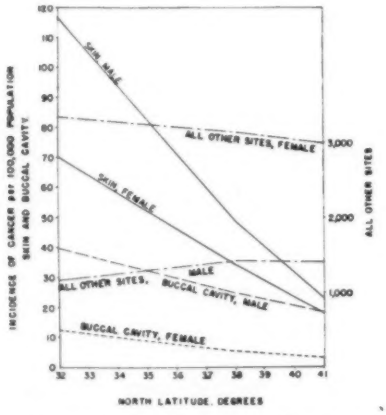

FIGURE 3.—Distribution of cancer incidence with respect to latitude. After data from Dorn (*27*). Reproduced from Actualités Medico-Chirurgicales, No. XIII by permission of the Belgian American Educational Foundation.

The factors determining the amount of carcinogenic radiation in sunlight must be understood in order to appreciate its dependence on latitude. The short wavelength limit of sunlight is determined by a layer of ozone located principally, if not entirely, in the stratosphere, which, if considered to be concentrated as pure gas at one atmosphere pressure, would be only 2 to 3 mm. thick. This tenuous layer absorbs wavelengths 3200A and shorter very strongly and consequently establishes the lower spectral limit of sunlight at about 2900A and protects the inhabitants of the earth from the destructive action of shorter wavelengths. The amount of absorption of the short ultraviolet radiation increases greatly as the sun moves away from the zenith, because the rays, instead of passing directly through the ozone layer at right angles, must pass through at a greater angle as referred to the zenith and hence through a greater thickness of ozone. This causes the carcinogenic radiation, i. e., wavelengths shorter than 3200A, to vary much more with season, time of day, and latitude than does the total solar radiation, the greater part of which is absorbed much less strongly by the atmosphere. (See (*15*).) The relationship of the carcinogenic radiation to total sunlight is suggested in figure 2; the relative variation with angle of the sun is illustrated by figure 2 in my earlier review (*1*), or figure 1 of (*15*). It is obvious, when these factors are considered, that any index based on total solar radiation (e. g., (*28*, *29*)) has little applicability in the present instance.

In order to make an accurate comparison between the incidence of carcinogenic radiation in sunlight at various latitudes and the corresponding incidence of cutaneous cancer, it would be necessary to know the action spectrum for carcinogenesis as well as to have complete data on the shorter wavelengths of sunlight. For reasons that have been previously discussed, it is infeasible to obtain the former data. Even if an accurate action spectrum could be obtained experimentally for the mouse (*6*), this would not apply quantitatively to human skin because of the difference in transmission of the ultraviolet radiation (*12*, *14*)

Direct measurements of the incidence of ultraviolet radiation have not been made at sufficient points on the earth to give a complete picture of their distribution, and it would be extremely laborious to do so; moreover, once obtained they would be of uncertain value in the solution of the present problem because their interpretation would depend upon the missing information regarding the wavelength dependence of carcinogenesis in human skin.

A general idea of this relationship can be obtained, however, from such data as are available. For the purpose, I have used those calculated by O'Brien (*30*) for antirachitic action (based on the absorption spectrum of provitamin D) which has the same long wavelength limit as carcinogenesis. There is no reason to believe that the action spectrum for carcinogenesis in man should be closely related to that for antirachitic action, and it might be predicted that the action spectrum for the erythema of sunburn would be closer to that to be expected for carcinogenesis. But there are inherent objections to using the accepted erythema spectrum for this purpose (*14*), and, in any case, O'Brien's calculations indicate that much the same relative values would be obtained as for the antirachitic spectrum. Such an estimate must be very rough at best, being influenced by numerous factors which cannot be readily taken into account, for example, cloudiness and dust. O'Brien's data for antirachitic action gives as satisfactory a rough index of carcinogenesis as is available, but it must be emphasized that no direct relationship between antirachitic action and carcinogenesis is implied in its use here.

In figure 4 the variation with latitude of both cancer incidence and carcinogenic radiation is indicated. For each variable the value for the lowest latitude (32°) has been taken as 100 percent for that variable in order to provide a relative basis for comparison. Plotted in this way, the incidence of cutaneous cancer shows about the same magnitude of change with latitude for both sexes. Two curves for annual incidence of carcinogenic radiation are plotted, one for 2.0 mm. ozone, and one for 2.8 mm. ozone; neither curve shows as great variation with latitude as do the cancer incidence curves. The ozone layer varies with latitude, and the two curves represent approximate extremes

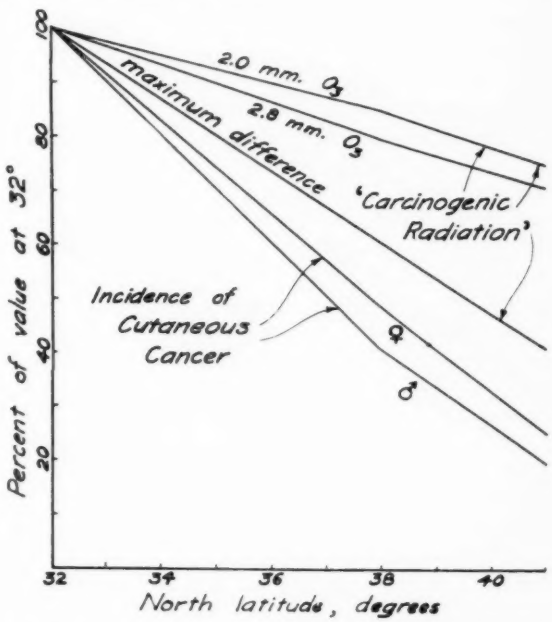

FIGURE 4.—Comparison of the variation with latitude, of cutaneous cancer incidence, and "carcinogenic" radiation. For each variable, the value for 32° is taken as 100 percent. Based on data of Dorn (*27*) for cancer incidence; and calculated distribution of "antirachitic" radiation by O'Brien (*30*).

for the latitudes covered by the cancer incidence data, 2.8 mm. representing the northernmost, 2.0 mm. the southernmost condition. Thus, the true range of variation with latitude should be greater than is indicated by either curve alone, and a third curve labeled "maximum difference" is introduced in which the annual ultraviolet radiation corresponding to 2.0 mm. ozone is used for latitude 32° and that for 2.8 mm. ozone is used for latitude 41°. The resulting curve has a slope more nearly resembling that of the cancer incidence curves, though not quite so steep; the agreement is certainly as good as is to be expected, considering the numerous assumptions that have had to be made in the analysis. The least that can be said is that the expected variation in the amount of carcinogenic radiation with latitude can be sufficient to account for the variation of cancer incidence with latitude as indicated by Dorn's data.

Such factors as amount of smoke and cloudiness are left out of consideration in the foregoing

analysis. These may be important in limiting the amount of carcinogenic radiation in specific areas, but we have no means for estimating their importance with regard to the present incidence data. There is another factor, which might help to explain the variation of cancer incidence with latitude. Rest periods from exposure to ultraviolet radiation lengthen the time to tumor appearance in mice (*21*, *25*), and this suggests that the longer winters of northern latitudes, with minimal incidence of carcinogenic radiation, constitute longer rest periods from this agent, enhancing the effect of latitude on cutaneous cancer incidence. The magnitude of such an effect cannot be accurately estimated, however.

It is possible, although we have no evidence therefor, that the heating of the skin by the sun enhances the carcinogenic action of the ultraviolet radiation, but sunlight is never intense enough to injure the skin by heating alone (*15*). There is little or no evidence to indicate the participation of other factors, such as wind, moisture, sweating, etc., all of which have been suggested as factors concerned in the incidence of cutaneous cancer.

RELATIVE IMMUNITY

Recent studies indicate that among Negroes cancers of the skin occur about as frequently on unexposed as on exposed parts (*31*, *32*). In the white population, in contrast, such tumors occur predominantly on the exposed parts. This lends strong support to the idea that the relative immunity of the Negro to cancer of the skin (e. g., see (*27*)) is explainable on the basis of his relative immunity to the action of sunlight, whereas this agent is responsible for the great majority of cutaneous cancers in the white population. The Negro is much less susceptible to sunburn, presumably because the effective wavelengths of ultraviolet radiation are largely absorbed before they reach the living epidermal cells (*15*), and his relative immunity to cancer may be reasonably attributed to the same cause.

PROPHYLAXIS

If we may accept, after summing all the evidence, the conclusion that sunlight is a major factor in human cutaneous cancer, the question arises what means may be devised for protection against this type of malignancy. Flight from sunlight would be a foolish thing to counsel since the basal-cell and squamous-cell carcinoma which we attribute to this agent are among the least dangerous of cancers, and their incidence is relatively low under conditions of their most frequent occurrence (fig. 3). Hence, it would seem inadvisable to instill great fear in this regard among the public in general, but at the same time a certain degree of caution is to be recommended. The evidence presented herein provides some basis for estimating the conditions which need to be avoided, namely, continuous year-round exposure to sunlight particularly in lower latitudes. Moreover, continuous regular exposures to artificial sources of ultraviolet radiation, even in moderate doses, would seem unwise, particularly when used during the winter months to supplement exposures during the summer either for "cosmetic" or "therapeutic" reasons. The fact that many persons may practice such procedure without ill effect is not evidence of its harmlessness, because only an occasional individual may be expected to develop cutaneous cancer under such conditions.

Some prophylaxis should be possible, since the dermatologist is able to recognize gross, "precancerous" changes in the skin, for instance, the appearance of keratoses, and it would seem wise from a practical point of view to protect skin which has shown such changes from further exposure to sunlight. Protection can be gained from application to the skin of materials which absorb ultraviolet radiation. Any such agent which gives good protection against sunburn should be efficacious, but while many have been devised, not all are of great value.

Unfortunately, in spite of extensive studies, no thoroughly satisfactory method for testing such materials has been developed, and some methods in use may lead to quite false conclusions (*33*). Spectroscopic determinations of transmission may indicate absurdly high protective value unless light scattering is properly taken into account, as Luckiesh et al. (*34*) have done by dispersing the incident beam. The problem of validating spectroscopic measurements in terms of protection of human skin against sunlight is a more difficult one. Blum, Eicher, and Terus (*33*) found that tests carried out on human skin indicated widely differ-

ent values for protection when different sources of radiation were used. For example, tests with carbon arc radiation always indicated much less protection against a given sunburn preventive than did tests with the mercury arc. The explanation lies in the physical characteristics of the skin and differences in spectral distribution of the radiation from these sources, which both, in turn, differ widely from sunlight (*33*). The necessary validation of tests for such preparations would have to be based upon extensive studies of exposure to sunlight itself, and this is a very difficult task. Giese and Wells (*35*, *36*) employed tests similar to those used by us (*33*) but report only results with mercury arc radiation. They do not state the individual variation, which we found to be high, nor validate their measurements in terms of sunlight. It appears, in general, that ointments containing a scattering agent such as titanium dioxide and an ultraviolet absorber such as phenyl-salicylate are the most efficacious (*36*, *39*). A preparation of this type appears in the National Formulary, 8th edition (1946).

CONCLUSION

At the end of my last review on this subject, I stated, "Much more exact data, both clinical and experimental, are required to place the concept on a thoroughly sound footing." If one weighs carefully the evidence that has accumulated since the writing of that statement, it seems today needlessly conservative. As stated in my introduction, this is the kind of problem to which an answer can only be found in terms of converging evidence from a number of points of view, for it is hard to conceive what single line of attack could provide proof in such a case. We have now a number of lines of evidence, all of which converge to indict sunlight as the major cause of cancer of the skin of man. This does not mean that sunlight is responsible for all types of cutaneous cancer; the malignant melanomas, for example, probably have very little or no connection with sunlight, as may be judged from their topographic distribution. On the other hand, the majority of cancers of the skin are of basal-cell or squamous-cell type, and most of these can be attributed with considerable certainty to the action of sunlight, specifically those ultraviolet wavelengths shorter than 3200A. Protection against such radiation should be feasible but must be devised with proper reference to the physics as well as the physiology of the problem.

REFERENCES

(*1*) Blum, H. F.: Sunlight and cancer of the skin. J. Nat. Cancer Inst. 1: 397–421 (1940).

(*2*) Funding, G., Henriques, O. M., and Rekling, E.: Über Lichtkanzer. 3rd Intern. Kongr. f. Lichtforschung, Wiesbaden, pp. 166–168 (1936).

(*3*) Preliminary report of Dritter Internationale Kongress für Lichtforschung. Strahlentherapie 61: 403–716 (1938).

(*4*) Roffo, A. H.: Cancer et soleil. Bull. Assoc. franç. pour l'étude du cancer 23: 590–616 (1934).

(*5*) Rusch, H. P., Kline, B. E., and Baumann, C. A.: Carcinogenesis by ultraviolet rays with reference to wavelength and energy. Arch. Path. 31: 135–146 (1941).

(*6*) Blum, H. F.: Wavelength dependence of tumor induction by ultraviolet radiation. J. Nat. Cancer Inst. 3: 533–537 (1943).

(*7*) Blum, H. F., and Lippincott, S. W.: Carcinogenic effectiveness of ultraviolet radiation of wavelength 2537A. J. Nat. Cancer Inst. 3: 211–216 (1942).

(*8*) Hollaender, A.: Effects of ultraviolet radiation. Ann. Rev. Physiol. 8: 1–16 (1946).

(*9*) Greenstein, J. P.: Biochemistry of cancer. Academic Press, N. Y. (1947).

(*10*) Bain, J. A., and Rusch, H. P.: Carcinogenesis and ultraviolet radiation of wavelength 2800–3400A. Cancer Research 3: 425–430 (1943).

(*11*) Blum, H. F., Kirby-Smith, J. S., and Grady, H. G.: Quantitative induction of tumors in mice with ultraviolet radiation. J. Nat. Cancer Inst. 2: 259–268 (1941).

(*12*) Kirby-Smith, J. S., Blum, H. F., and Grady, H. G.: Penetration of ultraviolet radiation into skin, as a factor in carcinogenesis. J. Nat. Cancer Inst. 2: 403–412 (1942).

(*13*) Blum, H. F.: Action spectra and absorption spectra. *In* Methods of Biophysical Research, edited by F. M. Uber. Interscience Publ., New York (in press).

(*14*) Blum, H. F., and Terus, W. S.: The erythemal threshold for sunburn. Am. J. Physiol. 146: 107–117 (1946).

(*15*) Blum, H. F.: The physiological effects of sunlight on man. Physiol. Rev. 25: 483–530 (1945).

(*16*) Bergmann, W., Stavely, H. E., Strong, L. C., and Smith, G. M.: Studies on the hypothetical carcinogenicity of irradiated sterols. I. The effects of irradiated cholesterol on the skin of mice. Am. J. Cancer 38: 81–85 (1940).

(17) BLUM, H. F.: Relationships beween spontaneous tumors of lung and cutaneous tumors induced with ultraviolet radiation in strain A mice. J. Nat. Cancer Inst. 5: 89–97 (1944).

(18) GRADY, H. G., BLUM, H. F., and KIRBY-SMITH, J. S.: Pathology of tumors of the external ear in mice induced by ultraviolet radiation. J. Nat. Cancer Inst. 2: 269–276 (1941).

(19) GRADY, H. G., BLUM, H. F., and KIRBY-SMITH, J. S.: Types of tumor induced by ultraviolet radiation and factors influencing their relative incidence. J. Nat. Cancer Inst. 3: 371–378 (1943).

(20) BLUM, H. F., and TERUS, W. S.: Inhibition of the erythema of sunburn by large doses of ultraviolet radiation. Am. J. Physiol. 146: 97–106 (1946).

(21) PACK, G. T.: A clinical study of pigmented nevi and melanomas. *In* The Biology of Melanomas. Special Publications of the N. Y. Acad. Sci. 4: 52–62 (1948).

(22) BLUM, H. F., GRADY, H. G., and KIRBY-SMITH, J. S.: Limits of accuracy in experimental carcinogenesis as exemplified by tumor induction with ultraviolet radiation. J. Nat. Cancer Inst. 3: 83–89 (1942).

(23) BLUM, H. F.: Some fundamental aspects of tumor development illustrated by studies with ultraviolet radiation. J. Nat. Cancer Inst. 3: 569–581 (1943).

(24) BLUM, H. F., GRADY, H. G., and KIRBY-SMITH, J. S.: Relationships between dosage and rate of tumor induction by ultraviolet radiation. J. Nat. Cancer Inst. 3: 91–97 (1942).

(25) BLUM, H. F.: Accuracy and reproductibility in the induction of tumors with ultraviolet radiation. J. Nat. Cancer Inst. 4: 75–79 (1943).

(26) BLUM, H. F.: Growth rate and development of tumors induced with ultraviolet radiation. J. Nat. Cancer Inst. 4: 559–569 (1944).

(27) DORN, H. F.: Illness from cancer in the United States. Pub. Health Rep. 59: 33–48, 65–77, 97–115 (1944).

(28) APPERLY, F. L.: The relation of solar radiation to cancer mortality in North America. Cancer Research 1: 191–195 (1941).

(29) SMITH, J. H.: The relation between deficiency of solar radiation and mortality due to pernicious anemia in the United States. Am. J. M. Sc. 188: 200–205 (1934).

(30) O'BRIEN, B.: Some biological effects of solar radiation. Ann. Rep. Smithsonian Instn.: 109–134 (1943).

(31) SCHREK, R.: Cutaneous carcinoma, IV. Analysis of 20 cases in negroes. Cancer Research 4: 119–127 (1944).

(32) ———: The racial distribution of cancer. I. Epithelial tumors of the skin, lip, and breast. Cancer Research 4: 433–437 (1944).

(33) BLUM, H. F., EICHER, M., and TERUS, W. S.: Evaluation of protective measures against sunburn. Am. J. Physiol. 146: 118–125 (1946).

(34) LUCKIESH, M., TAYLOR, A. H., COLE, H. N., and SOLLMAN, T.: Protective skin coatings for the prevention of sunburn. J. A. M. A. 130: 1–6 (1946).

(35) GIESE, A. C., and WELLS, J. M.: Sunburn protection, natural and artificial. Scient. Monthly 42: 458–464 (1946).

(36) GIESE, A. C., and WELLS, J. M.: Sweat- and water-resistant sunburn preparations. J. Am. Pharm. (Scientific ed.) 35: 208–212 (1946).

(37) FINDLAY, G. M.: Ultra-violet light and skin cancer. Lancet 2 (215): 1070–1073 (1928).

(38) BEARD, H. H., BOGGESS, T. S., and VON HAMM, E.: Experimental production of malignant tumors in the albino rat by means of ultraviolet rays. Am. J. Cancer 27: 257–266 (1936).

(39) BLUM, H. F.: Studies described in diver's reports of the Naval Medical Research Institute (1943–45).

ABSTRACTS

AMMONIA FORMATION FROM CYSTINE PEPTIDES AND DEHYDROPEPTIDES IN RAT-LIVER EXTRACTS, by *Jesse P. Greenstein* and *Florence M. Leuthardt*. Arch. Biochem. 18: 377–382 (1948).

The enzymic production of ammonia has been studied in rat-liver digests of diacetyl-*l*-cystine, dichloroacetyl-*l*-cystine, diglycyl-*l*-cystine, di-*dl*-chloropropinonyl-*l*-cystine, and di-*dl*-alanyl-*l*-cystine. The rate of this production has been compared under similar conditions with analogous peptides of *dl*-alanine, *dl*-serine, and dehydroalanine.

Dichloroacetyl-*l*-cystine, diglycyl-*l*-cystine, and di-*dl*-alanyl-*l*-cystine yield ammonia at nearly the same rate. Diacetyl-*l*-cystine is less suspectible, and di-*dl*-chloropropionyl-*l*-cystine yields no ammonia. Chloroacetyldehydroalanine is hydrolyzed at a rate considerably greater than that of acetyldehydroalanine and of *dl*-chloropropionyldehydroalanine, although these rates are much below those for glycyldehydroalanine and for *dl*-alanyldehydroalanine. No effect of hydrogen sulfide on the rate of hydrolysis of dehydropeptides in rat-liver homogenates has been observed. The possible paths of metabolism for the cystine peptides are discussed.

dl-Alanine, *dl*-serine, and their peptides yield little or no ammonia under conditions whereby peptides of cystine and of dehydroalanine are readily metabolized.

DERIVATIVES OF α,α-DI(GLYCYLAMINO)PROPIONIC ACID, by *Jesse P. Greenstein* and *Vincent E. Price*. J. Biol. Chem. 175: 963–967 (1948).

α,α-Di(N-methylglycylamino)propionic acid HCl, α,α-di(*N*-dimethylglycylamino) propionic acid HCl, α,α-di(chloroacetylglycylamino)propionic acid, and α,α-di (glycylglycylamino)propionic acid HCl were prepared, and their hydrolysis in various rat tissues was followed by the rate of ammonia evolution over the controls. The monomethylglycyl- and glycylglycyl-derivatives were hydrolyzed by all normal tissues studied, whereas the dimethylglycyl- and chloroacetylglycyl- derivatives were not attacked. In extracts of the hepatoma, the susceptibility of di(glycylamino)propionic acid is higher than in liver, whereas that of the di(*N*-methylglycylamino) propionic acid nearly vanishes. Comparison is made between analogously constituted dehydropeptides and di(glycylamino)propionic acid derivatives, and certain similarities are described.

ENZYMATIC HYDROLYSIS OF 2,4-DIKETO ACIDS, by *Alton Meister* and *Jesse P. Greenstein*. J. Biol. Chem. 175: 573–578 (1948).

It has been found that extracts of liver and kidney catalyze the hydrolysis of 2,4-diketovaleric acid, yielding nearly equivalent amounts of pyruvic and acetic acids. This reaction occurs aerobically and anaerobically and without detectable acetoacetate formation. The effect of substrate and liver-extract concentration on the rate of hydrolysis have been studied. Formation of pyruvate from 2,4-diketovaleric acid has also been demonstrated in liver slices. A series of 11 other 2,4-diketo acids is hydrolyzed in a similar manner to yield pyruvic acid. The hydrolysis of 2,4-diketoundecylic acid resulted in the appearance of equimolar amounts of pyruvic and octanoic acids. Of the diketo acids studied, 2,4-diketohexanoic and 2,4-diketocapric acids are hydrolyzed most rapidly.

The pH optima for hydrolysis of the normal aliphatic 2,4-diketo acids are in the range 7.2–7.9 for the 5, 6, and 7-carbon acids, and 8.0–8.9 for the acids containing between 8 and 11 carbon atoms. The term "acylpyruvase" is proposed for the enzyme or enzymes which catalyze 2,4-diketo acid hydrolysis. Acylpyruvase activity is present in the liver and kidney of the rat, rabbit, cat, and mouse. All other tissues studied, including primary rat hepatoma, possess little enzymatic activity. A partially purified enzyme preparation has been obtained from rat liver.

Methods of preparation of the disodium salts of 2,4-diketo acids are given. The ultraviolet absorption spectra for the ethyl esters of aliphatic and aromatic 2,4-diketo acids and the relationship between enolization and ultraviolet absorption are described. A spectrophotometric method for the determination of acylpyruvase activity is presented. Implications of these findings in terms of intermediary metabolism are discussed.

ACETYLATED DEHYDROAMINO ACIDS, by *Vincent E. Price* and *Jesse P. Greenstein*. Arch. Biochem. 18: 383–386 (1948).

Acetyldehydroaminobutyric acid, acetyldehydrovaline, acetyldehydronorleucine, acetyldehydroleucine, and acetyldehydroalanine were prepared, and their absorption spectra and susceptibility to enzymatic hydrolysis in rat-liver and kidney digests were studied. Acetyldehydroalanine possesses a specific absorption in the ultraviolet region and is readily metabolized. In contrast, the other compounds possess a general, high absorption in the ultraviolet and are apparently not metabolized. Acetyldehydrotyrosine and acetyldehydrophenylalanine are also not metabolized in these tissues. α-Ketoisocaproic acid was prepared by the hydrolysis of acetyldehydroleucine in hot HCl. Like pyruvic acid, addition of this keto acid to a rat-liver digest of glutamine at pH 6.8 produced an acceleration of desamidation of the glutamine.

ENZYMATIC HYDROLYSIS OF ANALOGOUS SATURATED AND UNSATURATED PEPTIDES, by *Vincent E. Price* and *Jesse P. Greenstein*. J. Biol. Chem. 175: 969–974 (1948).

The rates of hydrolysis in fresh, aqueous, rat-tissue extracts were determined for glycyl-*dl*-alanine,, glycyl-*dl*-phenylalanine, chloroacetyl-*dl*-alanine, acetyl-*dl*-alanine, and chloroacetyl-*dl*-phenylalanine, and the rates were compared both among themselves and with those of the corresponding analogous dehydropeptides. Both optical isomers of the glycyl peptides of alanine and phenylalanine were hydrolyzed, one at a faster rate than the other, but only one isomer of the chloroacetyl and acetyl peptides was hydrolyzed. Chloroacetylglycine was not hydrolyzed, and glycylglycine was hydrolyzed in kidney at a rate approximately one-fifteenth that of the more susceptible isomer or glycyl-*dl*-alanine.

The more susceptible isomers of the racemic saturated peptides were hydrolyzed in extracts of kidney, liver, and hepatoma at considerably faster rates than those of the corresponding dehydropeptides. The neoplastic transformation of liver results in a decrease in the hydrolysis rate of glycylalanine, which is in contrast with the increase in hydrolysis rate of glycyldehydroalanine. The more suspectible antipodes of the saturated peptides of phenylalanine were hydrolyzed in kidney, liver, and hepatoma at a faster rate than those of alanine, whereas the converse held true of the corresponding dehydropeptides. The question of the separate identity of the peptidases and dehydropeptidases is discussed.

STUDIES ON THE POSSIBLE MULTIPLE NATURE OF DEHYDROPEPTIDASE I, by *Jesse P. Greenstein, Vincent E. Price*, and *Florence M. Leuthardt*. J. Biol. Chem. 175: 953–962 (1948).

dl-Alanyldehydrophenylalanine, N-methyl-*dl*-alanyldehydrophenylalanine, N-methylglycyldehydrophenylalanine, chloroacetyl-*dl*-alanyldehydroalanine, chloroacetylglycyldehydroalanine, and glycylglycyldehydroalanine were synthesized and together with other dehydropeptides previously described were investigated in digests with various rat tissues.

Although chloroacetylalanyldehydroalanine is hydrolyzed by all the tissues studied, chloroacetylglycyldehydroalanine and chloroacetylglycyldehydrophenylalanine are resistant. Amination of the last two compounds to the corresponding glycyl derivatives restores susceptibility to enzymatic action. N-methylated dehydropeptides are hydrolyzed at a slower rate than the parent compounds.

The rate of hydrolysis of glycyldehydroalanine and of alanyldehydroalanine by the rat hepatoma is about sixfold and twentyfold that, respectively, of normal liver. Neither hepatoma nor brain appears to have any capacity to hydrolyze peptides of dehydrophenylalanine. The capacity of liver to hydrolyze N-methylglycyldehydroalanine diminishes when this tissue becomes neoplastic.

The pH-activity functions of the various dehydropeptides in digests of rat liver and kidney were determined and appeared to involve roughly three categories of substrates with optimum susceptibilities at pH 8.0, 8.8, and 9.0. The possible multiple nature of dehydropeptidase I is discussed.

CULPABILITY FOR THE DELAY IN THE MANAGEMENT OF CANCER, by *DeWitt DeLawter*. M. Ann. D. C. 17 (6): 342–347 (1948).

A review of the management of 420 cases of cancer treated at the Warwick Memorial Clinic, Washington, D. C., principally during 1947, suggested that progress is being made in reducing the delay in the handling of cancer cases as compared with a similar study made 10 years previously in New York City.

In this study, 37.4 percent of the cases were managed without undue delay either on the part of the patient or the physician as compared with 20.7 percent in the New York series.

The delay in the management of cancer after the diagnosis and before the onset of treatment was excessive in 45.7 percent of the cases studied. Some of the causes for this unusual delay are discussed.

THE DIFFUSION AND SEDIMENTATION OF SODIUM THYMONUCLEATE, by *Herbert Kahler*. J. Phys. & Colloid Chem. 52: 676–689 (1948).

The diffusion constant increases with concentration according to the relation

$$D = D_0(1 + k_d c)$$

In strong salt solution the value of k_d decreases. The Boltzmann equation is used to determine the trend of the diffusion constant with concentration and to estimate the diffusion for the smallest particles. It is shown that the distance of the mode from the starting point increases (negatively) approximately as $t^{\frac{1}{2}}$. It is shown that for a polydisperse system, if all components have a linear concentration dependence and the system has a constant polydispersity, the weight-average diffusion over all components is also linear.

The sedimentation is described by $1/s - 1/s_0 = kc$, the plot of $1/s$ against c giving a straight line, extrapolating to $1/s_0$. Sedimentation in the presence of 3 N potassium chloride gives a slightly higher velocity, and 0.09 M calcium chloride gives a velocity approaching the velocity for infinite dilution. The sedimentation in water at 0.2-percent nucleate concentration is about one-fourth the value in buffer.

It is shown that with constant polydispersity a linear relation between $1/s_1$ and c_1 leads to a linear relation for the weight-average reciprocal sedimentation constant. The molecular weight and shape factors were obtained. The relation between the molecular weights of nucleate and nucleohistone is discussed.

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